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ALBERTE, RANDALL S., University of Chicago, Barnes Laboratory, 5630 S. Ingleside Ave., Chicago, IL 60637
ALBRIGHT, JOHN T., 7 Siders Pond Rd., Falmouth, MA 02540
ALKON, DANIEL, Section on Neural Systems, Laboratory of Biophysics, NIH, Marine Biological Laboratory, Woods Hole, MA 02543
ALLEN, GARLAND E., Department of Biology, Washington University, St. Louis, MO 63130
ALLEN, NINA S., Department of Biology, Wake Forest University, Box 7325, Reynolds Station, Winston-Salem, NC 27109
ALLEN, ROBERT D., Department of Biology, Dartmouth College, Hanover, NH 03755
ALSCHER, RUTH, Department of Biology, Manhattanville College, Purchase, NY 10577
AMATNIEK, ERNEST, 4797 Boston Post Rd., Pelham Manor, NY 10803
ANDERSON, EVERETT, Department of Anatomy, LHRBB, Harvard Medical School, Boston, MA 02115
ANDERSON, J. M., Cornell University, Emerson Hall, Ithaca, NY 14850
ARMET-KIBEL, CHRISTINE, Biology Department, University of Massachusetts—Boston, Boston, MA 02125
ARMSTRONG, CLAY M., Department of Physiology, Medical School, University of Pennsylvania, Philadelphia, PA 19174

ARMSTRONG, PETER B., Department of Zoology, University of California, Davis, CA 95616
ARNOLD, JOHN M., Pacific Biomedical Research Center, University of Hawaii, 41 Ahui St., Honolulu, HI 96813
ARNOLD, WILLIAM A., 102 Balsam Rd., Oak Ridge, TN 37830
ASHTON, ROBERT W., Gaston Snow Beekman and Bogue, 14 Wall St., New York, NY 10005
ATEMA, JELLE, Marine Biological Laboratory, Woods Hole, MA 02543
ATWOOD, KIMBALL C., 100 Haven Ave., Apt. 21-E, New York, NY 10032
AUGUSTINE, GEORGE, JR., Department of Biology, University of California, Los Angeles, CA 90024
AUSTIN, MARY L., 506½ N. Indiana Ave., Bloomington, IN 47401
BACON, ROBERT, P.O. Box 723, Woods Hole, MA 02543
BAKER, ROBERT G., New York University Medical Center, 550 First Ave., New York, NY 10016
BALDWIN, THOMAS O., Department of Biochemistry and Biophysics, Texas A&M University, College Station, TX 77843
BALLOTTI, GENO A., Permanent Charity Fund of Boston, Boston Place, Boston, MA 02106
BANG, BETSY, 76 F. R. Lillie Rd., Woods Hole, MA 02543
BARKER, JEFFERY L., NIH, Bldg. 36 Room 2002, Bethesda, MD 20205
BARLOW, ROBERT B., JR., Institute for Sensory Research, Syracuse University, Merrill Lane, Syracuse, NY 13210
BARTELL, CLELMER K., 2000 Lake Shore Drive, New Orleans, LA 70122
BARTH, LUCENA J., 26 Quissett Ave., Woods Hole, MA 02543
BARTLETT, JAMES H., Department of Physics, Box 1921, University of Alabama, University, AL 35486
BATTELLE, BARBARA-ANNE, National Eye Institute, NIH, Bethesda, MD 20205
BAUER, G. ERIC, Department of Anatomy, University of Minnesota, Minneapolis, MN 55414
BEAUGE, LUIS ALBERTO, Instituto de Investigacion Medica, Casilla de Correo 389, 5000 Cordoba, Argentina
BECK, L. V., School of Experimental Medicine, Department of Pharmacology, Indiana University, Bloomington, IN 47401
BEGG, DAVID A., LHRRB, Harvard Medical School, 45 Shattuck St., Boston, MA 02115
BELL, EUGENE, Department of Biology, Massachusetts Institute of Technology, 77 Massachusetts Ave., Cambridge, MA 02139
BENNETT, M. V. L., Albert Einstein College of Medicine, Department of Neuroscience, 1300 Morris Park Ave., Bronx, NY 10461
BENNETT, MIRIAM F., Department of Biology, Colby College, Waterville, ME 04901
BERG, CARL J., JR., Marine Biological Laboratory, Woods Hole, MA 02543
BERNE, ROBERT W., University of Virginia, School of Medicine, Charlottesville, VA 22908
BERNHEIMER, ALAN W., New York University, College of Medicine, New York, NY 10016 (Life Member 10/83)
BEZANILLA, FRANCISCO, Department of Physiology, University of California, Los Angeles, CA 90052
BIGGERS, JOHN D., Department of Physiology, Harvard Medical School, Boston, MA 02115
BISHOP, STEPHEN H., Department of Zoology, Iowa State University, Ames, IA 50010
BODIAN, DAVID, Department of Otolaryngolgy, Johns Hopkins University, 1721 Madison St., Baltimore, MD 21206
BOETTIGER, EDWARD G., 29 Juniper Point, Woods Hole, MA 02543
BOGORAD, LAWRENCE, The Biological Laboratories, Harvard University, Cambridge, MA 02138
BOOLOOTIAN, RICHARD A., Science Software Systems, Inc., 11899 W. Pico Blvd., W. Los Angeles, CA 90064
BOREI, HANS G., Department of Zoology, University of Pennsylvania, Philadelphia, PA 19174
BORGESE, THOMAS A., Department of Biology, Lehman College, CUNY, Bronx, NY 10468
BORISY, GARY G., Laboratory of Molecular Biology, University of Wisconsin, Madison, WI 53715
BOSCH, HERMAN F., Whipple Hill, Richmond, NH 03470
BOWEN, DANIEL, Department of Biology, University of California, Santa Barbara, CA 93106
BOWEN, VAUGHN T., 652 Knox Rd., Strafford, Wayne, PA 19087

BOWLES, FRANCIS P., P.O. Box 674, Woods Hole, MA 02543
BOYER, BARBARA C., Department of Biology, Union College, Schnectady, NY 12308
BRINLEY, F. J., Neurological Disorders Program, NINCDS, 716 Federal Building, Bethesda, MD 20205
BROWN, JOEL E., Department of Ophthalmology, Box 8096 Sciences Center, Washington University, 660 S. Euclid Ave., St. Louis, MO 63110
BROWN, STEPHEN C., Department of Biological Sciences, SUNY, Albany, NY 12222
BUCK, JOHN B., NIH, Laboratory of Physical Biology, Room 112, Building 6 Bethesda, MD 20205
BURDICK, CAROLYN J., Department of Biology, Brooklyn College, Brooklyn, NY 11210
BURGER, MAX, Department of Biochemistry, Biocenter of the University of Basel, Klingelbergstrasse 70, CH-4056 Basel, Switzerland
BURKY, ALBERT, Department of Biology, University of Dayton, Dayton, OH 45649
BURSTYN, HAROLD LEWIS, 523 National Center, U. S. Geological Survey, Reston, VA 22092
BURSZTAJN, SHERRY, Neurology Department—Program in Neuroscience, Baylor College of Medicine, Houston, TX 77030
BUSH, LOUISE, 7 Snapper Lane, Falmouth, MA 02540
CANDELAS, GRACIELA C., Department of Biology, University of Puerto Rico, Rio Piedras, PR 00931
CARIELLO, LUCIO, Stazione Zoologica, Villa Comunale, Napoli, Italy
CARLSON, FRANCIS D., Department of Biophysics, Johns Hopkins University, Baltimore, MD 21218
CASE, JAMES, Department of Biological Sciences, University of California, Santa Barbara, CA 93106
CASSIDY, J. D., University of Illinois at Chicago Circle Department of Biological Sciences, Box 4348, Chicago, IL 60680
CEBRA, JOHN J., Department of Biology, Leidy Labs, G-6, University of Pennsylvania, Philadelphia, PA 19174
CHAET, ALFRED B., University of West Florida, Pensacola, FL 32504
CHAMBERS, EDWARD L., Department of Physiology and Biophysics, University of Miami, School of Medicine, P.O. Box 520875, Miami, FL 33152
CHANG, DONALD C., Department of Physiology, Baylor College of Medicine, 1200 Moursund, Houston, TX 77030
CHAPPELL, RICHARD L., Department of Biological Sciences, Hunter College, Box 210, 695 Park Ave., New York, NY 10021
CHAUNCEY, HOWARD H., 30 Falmouth St., Wellesley Hills, MA 02181
CHILD, FRANK M., Department of Biology, Trinity College, Hartford, CT 06106
CITKOWITZ, ELNA, 410 Livingston St., New Haven, CT 06511
CLARK, A. M., 48 Wilson Rd., Woods Hole, MA 02543
CLARK, ELOISE E., Vice President for Academic Affairs, Bowling Green State University, Bowling Green, OH 43403
CLARK, HAYS, 26 Deer Park Drive, Greenwich, CT 06830
CLARK, JAMES M., Shearson/American Express, 14 Wall St., New York, NY 10005
CLARK, WALLIS H., JR., Aquaculture Program, Room 243, Department of Animal Science, University of California, Davis, CA 95616
CLAUDE, PHILIPPA, Primate Center, Capitol Court, Madison, WI 53706
CLAYTON, RODERICK K., Cornell University, Section of Genetics, Development and Physiology, Ithaca, NY 14850
CLOWES, GEORGE H. A., JR., The Cancer Research Institute, 194 Pilgrim Rd., Boston, MA 02215
CLUTTER, MARY, Cellular and Physiological Biosciences Section, National Science Foundation, 1800 G St., NW, Washington, DC 20550
COBB, JEWELL P., President, California State University, Fullerton, CA 92634
COHEN, ADOLPH I., Department of Ophthalmology, School of Medicine, Washington University, 660 S. Euclid Ave., St. Louis, MO 63110
COHEN, CAROLYN, Rosenstiel Basic Medical Sciences Research Center, Brandeis University, Waltham, MA 02154

COHEN, LAWRENCE B., Department of Physiology, Yale University, 333 Cedar St., New Haven, CT 06510

COHEN, SEYMOUR S., Department of Pharmacological Science, SUNY, Stony Brook, NY 11790

COHEN, WILLIAM D., Department of Biological Sciences, Hunter College, 695 Park Ave., New York, NY 10021

COLE, JONATHAN J., Institute for Ecosystems Studies, Cary Arboretum, Millbrook, NY 12545

COLEMAN, ANNETTE W., Division of Biology and Medicine, Brown University, Providence, RI 02912

COLLIER, JACK R., Department of Biology, Brooklyn College, Brooklyn, NY 11210

COLLIER, MARJORIE McCANN, Biology Department, Saint Peter's College, Kennedy Boulevard, Jersey City, NJ 07306

COOK, JOSEPH A., The Edna McConnell Clark Foundation, 250 Park Ave., New York, NY 10017

COOPERSTEIN, S. J., University of Connecticut, School of Medicine, Farmington Ave., Farmington, CT 06032

CORLISS, JOHN O., Department of Zoology, University of Maryland, College Park, MD 20742

CORNELL, NEAL W., 6428 Bannockburn Drive, Bethesda, MD 20817

CORNMAN, IVOR, 10A Orchard St., Woods Hole, MA 02543

CORSON, DAVID WESLEY, JR., Laboratory of Sensory Physiology, Marine Biological Laboratory, Woods Hole, MA 02543

COSTELLO, WALTER J., College of Medicine, Ohio University, Athens, OH 45701

COUCH, ERNEST F., Department of Biology, Texas Christian University, Fort Worth, TX 76129

CREMER-BARTELS, Gertrud, Universitats Augenklinik, 44 Munster, West Germany

CRIPPA, MARCO, Faculte des Sciences, Université de Genève, 20, quai Ernest-Ansermet, Geneve 4, Switzerland

CROW, TERRY J., Department of Physiology, University of Pittsburgh, School of Medicine, Pittsburgh, PA 15261

CROWELL, SEARS, Department of Biology, Indiana University, Bloomington, IN 47401

DAIGNAULT, ALEXANDER T., W. R. Grace Company, 1114 Avenue of the Americas, New York, NY 10036

DAN, KATSUMA, Professor Emeritus, Tokyo Metropolitan Union, Meguro-ku, Tokyo, Japan

DAVID, JOHN R., Seeley G. Mudd Building, Room 504, 250 Longwood Ave., Boston MA 02115

DAVID, ROBERTA A., Seeley G. Mudd Building, Room 504, 250 Longwood Ave., Boston, MA 02115

DAVIS, BERNARD D., Bacterial Physiology Unit, Harvard Medical School, 25 Shattuck St., Boston, MA 02115

DAVIS, JOEL P., Seapuit, Inc., P.O. Box G, Osterville, MA 02655

DAW, NIGEL W., 78 Aberdeen Place, Clayton, MO 63105

DEGROOF, ROBERT C., RR#1 Box 343, Green Lane, PA 18054

DEHAAN, ROBERT L., Department of Anatomy, Emory University, Atlanta, GA 30322

DELANNEY, LOUIS E., Institute for Medical Research, 2260 Clove Drive, San Jose, CA 95128

DEPHILLIPS, HENRY A., JR., Department of Chemistry, Trinity College, Hartford, CT 06106

DETERRA, NOEL, Marine Biological Laboratory, Woods Hole, MA 02543

DETTBARN, WOLF-DIETRICH, Department of Pharmacology, School of Medicine, Vanderbilt University, Nashville, TN 37127

DEWEER, PAUL J., Department of Physiology, School of Medicine, Washington University, St. Louis, MO 63110

DISCHE, ZACHARIAS, Eye Institute, College of Physicians and Surgeons, Columbia University, 639 W. 165 St., New York, NY 10032

DIXON, KEITH E., School of Biological Sciences, Flinders University, Bedford Park, South Australia

DOWDALL, MICHAEL J., Department of Biochemistry, University Hospital and Medical School, Nottingham N672 UH, England, U. K.

DOWLING, JOHN E., The Biological Laboratories, Harvard University, 16 Divinity Ave., Cambridge, MA 02138

DuBois, Arthur Brooks, John B. Pierce Foundation Laboratory, 290 Congress Ave., New Haven, CT 06519

Dudley, Patricia L., Department of Biological Sciences, Barnard College, Columbia University, New York, NY 10027

Dunham, Philip B., Department of Biology, Syracuse University, Syracuse, NY 13210

Ebert, James D., Office of the President, Carnegie Institution of Washington 1530 P St., NW, Washington, DC 20008

Eckberg, William R., Department of Zoology, Howard University, Washington, DC 20059

Eckert, Roger O., Department of Zoology, University of California, Los Angeles, CA 90024

Edds, Kenneth T., Department of Anatomical Sciences, SUNY, Buffalo, NY 14214

Edds, Louise, College of Osteopathic Medicine, Grosvenor Hall, Ohio University, Athens, OH 45701

Eder, Howard A., Albert Einstein College of Medicine, 1300 Morris Park Ave. Bronx, NY 10461

Edwards, Charles, Department of Biological Sciences, SUNY, Albany, NY 12222

Egyud, Laszlo G., P.O. Box 342, Woods Hole, MA 02543

Ehrenstein, Gerald, NIH, Bethesda, MD 20205

Ehrlich, Barbara E., Department of Physiology, Albert Einstein College of Medicine, 1300 Morris Park Ave., Bronx, NY 10461

Eisen, Arthur Z., Chief of Division of Dermatology, Washington University, St. Louis, MO 63110

Elder, Hugh Young, Institute of Physiology, University of Glasgow, Glasgow, Scotland, U. K.

Elliott, Gerald F., The Open University Research Unit, Foxcombe Hall, Berkeley Rd., Boars Hill, Oxford, England, U. K.

Epel, David, Hopkins Marine Station, Pacific Grove, CA 93950

Epstein, Herman T., Department of Biology, Brandeis University, Waltham, MA 02154

Erulkar, Solomon D., 318 Kent Rd., Bala Cynwyd, PA 19004

Essner, Edward S., Kresge Eye Institute, Wayne State University, 540 E. Canfield Ave., Detroit, MI 48201

Failla, Patricia M., Office of the Director, Argonne National Laboratory, Argonne, IL 60439

Farmanfarmaian, A., Department of Physiology and Biochemistry, Rutgers University, New Brunswick, NJ 08903

Faust, Robert G., Department of Physiology, Medical School, University of North Carolina, Chapel Hill, NC 27514

Fein, Alan, Laboratory of Sensory Physiology, Marine Biological Laboratory Woods Hole, MA 02543

Feldman, Susan C., Department of Anatomy, University of Medicine and Dentistry of New Jersey, New Jersey Medical School, 100 Bergen St., Newark, NJ 07103

Ferguson, F. P., National Institute of General Medical Science, NIH, Bethesda, MD 20205

Fessenden, Jane, Marine Biological Laboratory, Woods Hole, MA 02543

Finkelstein, Alan, Albert Einstein College of Medicine, 1300 Morris Park Ave., Bronx, NY 10461

Fischbach, Gerald, Department of Anatomy and Neurobiology, Washington University School of Medicine, St. Louis, MO 63110

Fischman, Donald A., Department of Cell Biology and Anatomy, Cornell University Medical College, 1300 York Ave., New York, NY 10021

Fisher, J. Manery, Department of Biochemistry, University of Toronto, Toronto, Ontario, Canada M5S 1A8

Fishman, Harvey M., Department of Physiology, University of Texas Medical Branch, Galveston, TX 77550

Flanagan, Dennis, Scientific American, 415 Madison Ave., New York, NY 10017

Fox, Maurice S., Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02138

Franzini, Clara, Department of Biology G-5, School of Medicine, University of Pennsylvania, Philadelphia, PA 19174

FRAZIER, DONALD T., Department of Physiology and Biophysics, University of Kentucky Medical Center, Lexington, KY 40536

FREEMAN, ALAN R., Department of Physiology, Temple University, 3420 N. Broad St., Philadelphia, PA 19140

FREEMAN, GARY L., Department of Zoology, University of Texas, Austin, TX 78172

FRENCH, ROBERT J., Department of Biophysics, University of Maryland, School of Medicine, Baltimore, MD 21201

FREYGANG, WALTER J., JR., 6247 29th St., NW, Washington, DC 20015

FULTON, CHANDLER, M., Department of Biology, Brandeis University, Waltham, MA 02154

FURSHPAN, EDWIN J., Department of Neurophysiology, Harvard Medical School, Boston, MA 02115

FUSELER, JOHN W., Department of Cell Biology, University of Texas Medical Branch, 53233 Harry Hines Blvd., Dallas, TX 75235

FUTRELLE, ROBERT P., Department of Genetics and Development, 515 Morrill Hall, University of Illinois, 505 S. Goodwin Ave., Urbana, IL 68101

FYE, PAUL, P.O. Box 309, Woods Hole, MA 02543

GABRIEL, MORDECAI, Department of Biology, Brooklyn College, Brooklyn, NY 11210

GAINER, HAROLD, Section of Functional Neurochemistry, NIH, Bldg. 36 Room 2A21, Bethesda, MD 20205

GALATZER-LEVY, ROBERT M., Room 1819, 55 East Washington Street, Chicago, IL 60602

GALL, JOSEPH G., Carnegie Institution, 115 West University Parkway, Baltimore, MD 21210

GASCOYNE, PETER, Marine Biological Laboratory, Woods Hole, MA 02543

GELFANT, SEYMOUR, Department of Dermatology, Medical College of Georgia, Augusta, GA 30904

GELPERIN, ALAN, Department of Biology, Princeton University, Princeton, NJ 08540

GERMAN, JAMES L., III, The New York Blood Center, 310 East 67th St., New York, NY 10021

GIBBS, MARTIN, Institute for Photobiology of Cells and Organelles, Brandeis University, Waltham, MA 02154

GIBSON, A. JANE, Wing Hall, Cornell University, Ithaca, NY 14850

GIFFORD, PROSSER, The Wilson Center, Smithsonian Building, 1000 Jefferson Drive, SW, Washington, DC 20590

GILBERT, DANIEL L., NIH, Laboratory of Biophysics, NINCDS, Bldg. 36, Room 2A-29, Bethesda, MD 20205

GIUDICE, GIOVANNI, Via Archirafi 22, Palermo, Italy

GLUSMAN, MURRAY, Department of Psychiatry, Columbia University, 722 W. 168th St., New York, NY 10032

GOLDEN, WILLIAM T., 40 Wall St., New York, NY 10005

GOLDMAN, DAVID E., 63 Loop Rd., Falmouth, MA 02540

GOLDMAN, ROBERT D., Department of Cell Biology and Anatomy, Northwestern University, 303 E. Chicago Ave., Chicago, IL 60611

GOLDSMITH, PAUL K., 5511 Oakmont Avenue, Bethesda, MD 20034

GOLDSMITH, TIMOTHY H., Department of Biology, Yale University, New Haven, CT 06520

GOLDSTEIN, MOISE H., JR., Johns Hopkins University, School of Medicine, 720 Rutland Ave., Baltimore, MD 21205

GOODMAN, LESLEY JEAN, Department of Zoology and Comparative Physiology, Queen Mary College, Mile End Road, London, E1 4NS, England, U. K.

GOTTSCHALL, GERTRUDE Y., 315 E. 68th St., 9-M, New York, NY 10021

GOUDSMIT, ESTHER, M., Department of Biology, Oakland University, Rochester, MI 48063

GOULD, ROBERT MICHAEL, Institute for Basic Research in Developmental Disabilities, 1050 Forest Hill Rd., Staten Island, NY 10314

GOULD, STEPHEN J., Museum of Comparative Zoology, Harvard University, Cambridge, MA 02138

GOVIND, C. K., Zoology Department—Scarborough, University of Toronto, 1265 Military Trail, West Hill, Ontario, Canada, M1C 1A4

GRANT, PHILIP, Department of Biology, University of Oregon, Eugene, OR 97403

GRASS, ALBERT, The Grass Foundation, 77 Reservoir Rd., Quincy, MA 02170

GRASS, ELLEN R., The Grass Foundation, 77 Reservoir Rd., Quincy, MA 02170
GRASSLE, JUDITH, Marine Biological Laboratory, Woods Hole, MA 02543
GREEN, JONATHAN P., Department of Biology, Roosevelt University, 430 S. Michigan Avenue, Chicago, IL 60605
GREENBERG, EVERETT PETER, Department of Microbiology, Stocking Hall, Cornell University, Ithaca, NY 14853
GREENBERG, MICHAEL J., C. V. Whitney Laboratory, Rt. 1, Box 121, St. Augustine, FL 32086
GREIF, ROGER L., Department of Physiology, Cornell University, Medical College New York, NY 10021
GRIFFIN, DONALD R., The Rockefeller University, 1230 York Ave., New York, NY 10021
GROSCH, DANIEL S., Department of Genetics, Gardner Hall, North Carolina State University, Raleigh, NC 27607
GROSS, PAUL R., President and Director, Marine Biological Laboratory, Woods Hole, MA 02543
GROSSMAN, ALBERT, New York University, Medical School, New York, NY 10016
GUNNING, A. ROBERT, P.O. Box 165, Falmouth, MA 02541
GWILLIAM, G. P., Department of Biology, Reed College, Portland, OR 97202
HALL, LINDA M., Department of Genetics, Albert Einstein College of Medicine, 1300 Morris Park Ave., Bronx, NY 10461
HALL, ZACK W., Department of Physiology, University of California, San Francisco, CA 94143
HALVORSON, HARLYN O., Rosenstiel Basic Medical Sciences Research Center, Brandeis University, Waltham, MA 02154
HAMLETT, NANCY VIRGINIA, Department of Biology, Swarthmore College, Swarthmore, PA 19081
HANNA, ROBERT B., College of Environmental Science and Forestry, SUNY, Syracuse, NY 13210
HARDING, CLIFFORD V., JR., Kresge Eye Institute, Wayne State University, 540 E. Canfield, Detroit, MI 48201
HAROSI, FERENC I., Laboratory of Sensory Physiology, Marine Biological Laboratory, Woods Hole, MA 02543
HARRIGAN, JUNE F., Laboratory of Biophysics, Marine Biological Laboratory, Woods Hole, MA 02543
HARRINGTON, GLENN W., Department of Microbiology, School of Dentistry, University of Missouri, 650 E. 25th St., Kansas City, MO 64108
HASCHEMEYER, AUDREY E. V., Department of Biological Sciences, Hunter College, 695 Park Ave., New York, NY 10021
HASTINGS, J. W., The Biological Laboratories, Harvard University, Cambridge, MA 02138
HAYASHI, TERU, 7105 SW 112 Pl., Miami, FL 33173
HAYES, RAYMOND L., JR., Howard University, College of Medicine, 520 W St., NW, Washington, DC 20059
HENLEY, CATHERINE, 5225 Pooks Hill Rd., #1127 North, Bethesda, MD 20034
HERNDON, WALTER R., University of Tennessee, 506 Andy Holt Tower, Knoxville, TN 37916
HESSLER, ANITA Y., 5795 Waverly Ave., La Jolla, CA 92037
HEUSER, JOHN, Department of Biophysics, Washington University, School of Medicine, St. Louis, MO 63110
HIATT, HOWARD H., Harvard University, School of Public Health, 677 Huntington Ave., Boston, MA 02115
HIGHSTEIN, STEPHEN M., Department of Otolaryngology, Washington University, St. Louis, MO 63110
HILDEBRAND, JOHN G., Department of Biological Sciences, Fairchild Center #913, Columbia University, New York, NY 10027
HILLIS-COLINVAUX, LLEWELLYA, Department of Zoology, The Ohio State University 484 W 12th Ave., Columbus, OH 43210
HILLMAN, PETER, Department of Biology, Hebrew University, Jerusalem, Israel
HINEGARDNER, RALPH T., Division of Natural Sciences, University of California, Santa Cruz, CA 95064

HINSCH, GERTRUDE, W., Department of Biology, University of South Florida, Tampa, FL 33620
HOBBIE, JOHN E., Ecosystems Center, Marine Biological Laboratory, Woods Hole, MA 02543
HODGE, ALAN J., Marine Biological Laboratory, Woods Hole, MA 02543
HODGE, CHARLES, IV, P.O. Box 4095, Philadelphia, PA 19118
HOFFMAN, JOSEPH, Department of Physiology, School of Medicine, Yale University, New Haven, CT 06515
HOFFMAN, RICHARD J., Department of Zoology, Iowa State University, Ames, IA 50011
HOLLYFIELD, JOE G., Baylor School of Medicine, Texas Medical Center, Houston, TX 77030
HOLTZMAN, ERIC, Department of Biological Sciences, Columbia University, New York, NY 10017
HOLZ, GEORGE G., JR., Department of Microbiology, SUNY, Syracuse, NY 13210
HOSKIN, FRANCIS C. G., Department of Biology, Illinois Institute of Technology, Chicago, IL 60616
HOUGHTON, RICHARD A., III, Ecosystems Center, Marine Biological Laboratory, Woods Hole, MA 02543
HOUSTON, HOWARD E., 2500 Virginia Ave., NW, Washington, DC 20037
HOWARTH, ROBERT, Ecosystems Center, Marine Biological Laboratory, Woods Hole, MA 02543
HOY, RONALD R., Section of Neurobiology and Behavior, Cornell University, Ithaca, NY 14850
HUBBARD, RUTH, The Biological Laboratories, Harvard University, Cambridge, MA 02138
HUFNAGEL, LINDA A., Department of Microbiology, University of Rhode Island, Kingston, RI 02881
HUMMON, WILLIAM D., Department of Zoology, Ohio University, Athens, OH 45701
HUMPHREYS, SUSIE H., Kraft Research and Development, 801 Waukegan Rd., Glenview, IL 60025
HUMPHREYS, TOM D., University of Hawaii, PBRC, 41 Ahui St., Honolulu, HI 96813
HUNTER, BRUCE W., Box 321, Lincoln Center, MA 01773
HUNTER, ROBERT D., Department of Biological Sciences, Oakland University, Rochester, NY 48063
HUNZIKER, HERBERT E., Esq., P.O. Box 547, Falmouth, MA 02541
HURWITZ, CHARLES, Basic Science Research Lab, Veterans Administration Hospital, Albany, NY 12208
HURWITZ, JERARD, Albert Einstein College of Medicine, Department of Molecular Biology, 1300 Morris Park Avenue, Bronx, NY 10461
HUXLEY, HUGH E., Medical Research Council, Laboratory of Molecular Biology, Cambridge, England, U. K.
HYNES, THOMAS J., JR., Meredith and Grew, Inc., 125 High Street, Boston, MA 02110
ILAN, JOSEPH, Department of Anatomy, Case Western Reserve University, Cleveland, OH 44106
INGOGLIA, NICHOLAS, Department of Physiology, New Jersey Medical School, 100 Bergen St., Newark, NJ 07103
INOUÉ, SADUYKI, McGill University Cancer Centre, Department of Anatomy, 3640 University St., Montreal, PQ, H3A 2B2, Canada
INOUÉ, SHINYA, Marine Biological Laboratory, Woods Hole, MA 02543
ISENBERG, IRVING, Department of Biochemistry and Biophysics, Oregon State University, Corvallis, OR 97331
ISSADORIDES, MARIETTA R., Department of Psychiatry, University of Athens, Monis Petraki 8, Athens, 140, Greece
ISSELBACHER, KURT J., Massachusetts General Hospital, Boston, MA 02114
IZZARD, COLIN S., Department of Biological Sciences, SUNY, Albany, NY 12222
JACOBSON, ANTONE G., Department of Zoology, University of Texas, Austin, TX 78712
JAFFE, LIONEL, Marine Biological Laboratory, Woods Hole, MA 02543
[illegible]HAN-PARWAR, BEHRUS, Worcester Foundation for Experimental Biology, 222 Maple Ave., Shrewsbury, MA 01545
[illegible]SCH, HOLGER W., Woods Hole Oceanographic Institution, Woods Hole, MA 02543

JEFFERY, WILLIAM R., Department of Zoology, University of Texas, Austin, TX 78712
JENNER, CHARLES E., Department of Zoology, University of North Carolina, Chapel Hill, NC 27514
JONES, MEREDITH L., Division of Worms, Museum of Natural History, Smithsonian Institution, Washington, DC 20560
JOSEPHSON, ROBERT K., School of Biological Sciences, University of California, Irvine, CA 92664
KABAT, E. A., Department of Microbiology, College of Physicians and Surgeons Columbia University, 630 West 168th St., New York, NY 10032
KALEY, GABOR, Department of Physiology, Basic Sciences Building, New York Medical College, Valhalla, NY 10595
KALTENBACH, JANE, Department of Biological Sciences, Mount Holyoke College, South Hadley, MA 01075
KAMINER, BENJAMIN, Department of Physiology, School of Medicine, Boston University, 80 East Concord St., Boston, MA 02118
KAMMER, ANN E., Division of Biology, Kansas State University, Manhatten, KS 66506
KANE, ROBERT E., University of Hawaii, PBRC, 41 Ahui St., Honolulu, HI 96813
KANESHIRO, EDNA S., Department of Biological Sciences, University of Cincinnati, Cincinnati, OH 45221
KAPLAN, EHUD, The Rockefeller University, 1230 York Ave., New York, NY 10021
KARAKASHIAN, STEPHEN J., Apt. 16-F, 165 West 91st St., New York, NY 10024
KARUSH, FRED, Department of Microbiology, School of Medicine, University of Pennsylvania, Philadelphia, PA 19174
KATZ, GEORGE M., Fundamental and Experimental Research, Merck, Sharpe and Dohme Rahway, NJ 07065
KEAN, EDWARD L., Department of Ophthalmology and Biochemistry, Case Western Reserve University, Cleveland, OH 44101
KELLEY, DARCY BRISBANE, Department of Biological Sciences, 1018 Fairchild, Columbia University, New York, NY 10032
KELLY, ROBERT E., Department of Anatomy, College of Medicine, University of Illinois, P.O. Box 6998, Chicago, IL 60680
KEMP, NORMAN E., Department of Zoology, University of Michigan, Ann Arbor, MI 48104
KENDALL, JOHN P., Faneuil Hall Associates, One Boston Place, Boston, MA 02108
KEYNAN, ALEXANDER, Hebrew University, Jerusalem, Israel
KINGSBURY, JOHN M., Department of Botany, Cornell University, Ithaca, NY 14853
KIRSCHENBAUM, DONALD, Department of Biochemistry, SUNY, 450 Clarkson Ave., Brooklyn, NY 11203
KLEIN, MORTON, Department of Microbiology, Temple University, Philadelphia, PA 19122
KLOTZ, I. M., Department of Chemistry, Northwestern University, Evanston, IL 60201
KOIDE, SAMUEL S., Population Council, The Rockefeller University, 66th St. and York Ave., New York, NY 10021
KONIGSBERG, IRWIN R., Department of Biology, Gilmer Hall, University of Virginia, Charlottesville, VA 22903
KORNBERG, HANS, Department of Biochemistry, University of Cambridge, Tennis Court Rd., Cambridge, CB2 7QW, England, U. K.
KOSOWER, EDWARD M., Ramat-Aviv, Tel Aviv, 69978 Israel
KRAHL, M. E., 2783 W. Casas Circle, Tucson, AZ 85741
KRANE, STEPHEN M., Massachusetts General Hospital, Boston, MA 02114
KRASSNER, STUART M., Department of Developmental and Cell Biology, University of California, Irvine, CA 92717
KRAUSS, ROBERT, FASEB, 9650 Rockville Pike, Bethesda, MD 20205
KRAVITZ, EDWARD A., Department of Neurobiology, Harvard Medical School, 25 Shattuck St., Boston, MA 02115
KRIEBEL, MAHLON E., Department of Physiology, B.S.B., Upstate Medical Center, 766 Irving Ave., Syracuse, NY 13210
KRIEG, WENDELL J. S., 1236 Hinman, Evanston, IL 60602

KRISTAN, WILLIAM B., JR., Department of Biology B-022, University of California San Diego, San Diego, CA 92093

KUHNS, WILLIAM J., University of North Carolina, 512 Faculty Lab Office, Bldg. 231-H, Chapel Hill, NC 27514

KUSANO, KIYOSHI, Illinois Institute of Technology, Department of Biology, 3300 South Federal St., Chicago, IL 60616

LADERMAN, AIMLEE, P.O. Box 689, Woods Hole, MA 02543

LAMARCHE, PAUL H., Eastern Maine Medical Center, 489 State St., Bangor, ME 04401

LANDIS, DENNIS M. D., Department of Neurology, Massachusetts General Hospital, Boston, MA 02114

LANDIS, STORY C., Department of Neurobiology, Harvard Medical School, Boston, MA 02115

LANDOWNE, DAVID, Department of Physiology, University of Miami, R-430, P.O. Box 016430, Miami, FL 33101

LANGFORD, GEORGE M., Department of Physiology, Medical Sciences Research Wing 206H, University of North Carolina, Chapel Hill, NC 27514

LASEK, RAYMOND J., Case Western Reserve University, Department of Anatomy, Cleveland, OH 44106

LASTER, LEONARD, University of Oregon, Health Sciences Center, Portland, OR 97201

LAUFER, HANS, Biological Sciences Group U-42, University of Connecticut, Storrs, CT 06268

LAUFFER, MAX A., Department of Biophysics, University of Pittsburgh, Pittsburgh, PA 15260

LAZAROW, JANE, 221 Woodlawn Ave., St. Paul, MN 55105

LAZARUS, MAURICE, Federated Department Stores, Inc., 50 Cornhill, Boston, MA 02108

LEADBETTER, EDWARD R., Biological Sciences Group U-42, University of Connecticut, Storrs, CT 06268

LEDERBERG, JOSHUA, President, The Rockefeller University, New York, NY 10021

LEDERHENDLER, IZJA I., Laboratory of Biophysics, Marine Biological Laboratory, Woods Hole, MA 02543

LEE, JOHN J., Department of Biology, City College of CUNY, Convent Ave. and 138th St., New York, NY 10031

LEFEVRE, PAUL G., Box 339, Shoreham, NY 11786

LEIBOVITZ, LOUIS, Laboratory for Marine Animal Health, Marine Biological Laboratory, Woods Hole, MA 02543

LEIGHTON, JOSEPH, 1201 Waverly Rd., Gladwyne, PA 19035

LEIGHTON, STEPHEN, NIH, Bldg. 13 3W13, Bethesda, MD 20205

LENHER, SAMUEL, 50-C Cokesbury Village, Hockessin, DE 19707

LERMAN, SIDNEY, Laboratory for Ophthalmic Research, Emory University, Atlanta, GA 30322

LERNER, AARON B., Yale University, School of Medicine, New Haven, CT 06510

LEVIN, JACK, Clinical Pathology Service, VA Hospital-113A, 4150 Clement St., San Francisco, CA 94120

LEVINTHAL, CYRUS, Department of Biological Sciences, Columbia University, 908 Schermerhorn Hall, New York, NY 10027

LEVITAN, HERBERT, Department of Zoology, University of Maryland, College Park, MD 20742

LING, GILBERT, 307 Berkeley Road, Marion, PA 19066

LIPICKY, RAYMOND J., Laboratory of Biophysics, NIH, Bldg. 36 Room 2A29, Bethesda, MD 20205

LISMAN, JOHN E., Department of Biology, Brandeis University, Waltham, MA 02154

LIUZZI, ANTHONY, Department of Physics, University of Lowell, Lowell, MA 01854

LLINAS, RODOLFO R., Department of Physiology and Biophysics, New York University Medical Center, 550 First Ave., New York, NY 10016

LOEWENSTEIN, WERNER R., Department of Physiology and Biophysics, University of Miami, P.O. Box 016430, Miami, FL 33101

LOEWUS, FRANK, A., Department of Argicultural Chemistry, Washington State University, Pullman, WA 99164

LOFTFIELD, ROBERT B., Department of Biochemistry, School of Medicine, University of New Mexico, 900 Stanford, NE, Alburquerque, NM 87105

LONDON, IRVING M., Massachusetts Institute of Technology, Cambridge, MA 02139

LONGO, FRANK J., Department of Anatomy, University of Iowa, Iowa City, IA 52442
LORAND, LASZLO, Department of Biochemistry and Molecular Biology, Northwestern University, Evanston, IL 60201
LURIA, SALVADOR E., Massachusetts Institute of Technology, Department of Biology, Cambridge, MA 02139
LYNCH, CLARA J., 4800 Fillmore Ave., Alexandria, VA 22311
MACAGNO, EDUARDO R., 1003B Fairchild, Columbia University, New York, NY 10022
MACNICHOL, E. F., JR., Laboratory of Sensory Physiology, Marine Biological Laboratory, Woods Hole MA 02543
MAGLOTT, DONNA R. S., Department of Zoology, Howard University, Washington, DC 20059
MAINER, ROBERT, The Boston Company, One Boston Place, Boston, MA 02108
MALKIEL, SAUL, Allergic Diseases, Inc., 130 Lincoln St., Worcester, MA 01605
MANALIS, RICHARD S., RR #10, 400N, Columbia City, IN 47625
MANGUM, CHARLOTTE P., Department of Biology, College of William and Mary, Williamsburg, VA 23185
MARGULIS, LYNN, Department of Biology, Boston University, 2 Cummington St., Boston, MA 02215
MARINUCCI, ANDREW C., Department of Civil Engineering, Princeton University, Princeton, NJ 08544
MARSH, JULIAN B., Department of Biochemistry and Physiology, Medical College of Pennsylvania, 3300 Henry Ave., Philadelphia, PA 19129
MARTIN, LOWELL V., Marine Biological Laboratory, Woods Hole, MA 02543
MASER, MORTON, 100 Hackmatak Way, Falmouth, MA 02540
MASTROIANNI, LUIGI, JR., Department of Obstetrics and Gynecology, University of Pennsylvania, Philadelphia, PA 19174
MATHEWS, RITA, W., c/o A. J. Johnson, New York University Medical Center, 550 First Ave., New York, NY 10016
MATTESON, DONALD R., Department of Physiology, G4, School of Medicine, University of Pennsylvania, Philadelphia, PA 19104
MAUTNER, HENRY G., Department of Biochemistry and Pharmacology, Tufts University, 136 Harrison Ave., Boston, MA 02111
MAUZERALL, DAVID, The Rockefeller University, 1230 York Ave., New York, NY 10021
MAZIA, DANIEL, Hopkins Marine Station, Pacific Grove, CA 93950
MCCANN, FRANCES, Department of Physiology, Dartmouth Medical School, Hanover, NH 03755
MCCLOSKY, LAWRENCE R., Department of Biology, Walla Walla College, College Place, WA 99324
MCLAUGHLIN, JANE A., P.O. Box 187, Woods Hole, MA 02543
MCMAHON, ROBERT F., Department of Biology, Box 19498, University of Texas, Arlington, TX 76019
MEEDEL, THOMAS, Boston University Marine Program, Marine Biological Laboratory, Woods Hole, MA 02543
MEINERTZHAGEN, IAN A., Department of Psychology, Life Sciences Center, Dalhousie University, Halifax, Nova Scotia, B3H 451, Canada
MEINKOTH, NORMAN A., Department of Biology, Swarthmore College, Swarthmore, PA 19081
MEISS, DENNIS E., Department of Biology, Clark University, Worcester, MA 01610
MELILLO JERRY A., Ecosystems Center, Marine Biological Laboratory, Woods Hole, MA 02543
MELLON, DEFOREST, JR., Department of Biology, University of Virginia, Charlottesville, VA 22903
MELLON, RICHARD P., P.O. Box 187, Laughlintown, PA 15655
MENZEL, RANDOLF, Institut fir Tierphysiologie, Free Universitat of Berlin, 1000 Berlin 41, Federal Republic of Germany
METUZALS, JANIS, Department of Anatomy, Faculty of Medicine, University of Ottawa, Ottawa, Ontario, K1N 9A9, Canada
METZ, CHARLES B., Institute of Molecular and Cellular Evolution, University of Miami, 521 Anastasia Ave., Carol Gables, FL 33134

MILKMAN, ROGER, Department of Zoology, University of Iowa, Iowa City, IA 52242
MILLS, ERIC L., Institute of Oceanography, Dalhousie University, Halifax, Nova Scotia
MILLS, ROBERT, 56 Worcester Court, Falmouth, MA 02540
MITCHELL, RALPH, Pierce Hall, Harvard University, Cambridge, MA 02138
MIYAMOTO, DAVID M., Department of Biology, Seton Hall University, South Orange, NJ 07079
MIZELL, MERLE, Department of Biology, Tulane University, New Orleans, LA 70118
MONROY, ALBERTO, Stazione Zoologica, Villa Communale, Napoli, Italy
MOORE, JOHN W., Department of Physiology, Duke University Medical Center, Durham, NC 27710
MOORE, LEE E., Department of Physiology and Biophysics, University of Texas, Medical Branch, Galveston, TX 77550
MORAN, JOSEPH F., JR., 23 Foxwood Drive, RR #1, Eastham, MA 02642 (resigned 10/83)
MORIN, JAMES G., Department of Biology, University of California, Los Angeles, CA 90024
MORRELL, FRANK, Department of Neurological Sciences, Rush Medical Center, 1753 W. Congress Parkway, Chicago, IL 60612
MORRILL, JOHN B., JR., Division of National Sciences, New College, Sarasota, FL 33580
MORSE, RICHARD S., 193 Winding River Rd., Wellesley, MA 02181
MORSE, ROBERT W., Box 574, N. Falmouth, MA 02556
MORSE, STEPHEN SCOTT, Department of Biological Sciences, Rutgers University, Nelson Biological Laboratories, New Brunswick, NJ 08903
MOSCONA, A. A., Department of Biology, University of Chicago, 920 East 58th St., Chicago, IL 60637
MOTE, MICHAEL I., Department of Biology, Temple University, Philadelphia, PA 19122
MOUNTAIN, ISABEL, Vinson Hall #112, 6251 Old Dominion Drive, McLean, VA 22101
MUSACCHIA, XAVIER J., Graduate School, University of Louisville, Louisville, KY 40292
NABRIT, S. M., 686 Beckwith St., SW, Atlanta, GA 30314
NAKA, KEN-ICHI, National Institute for Basic Biology, Okazaki 444, Japan
NAKAJIMA, SHIGEHIRO, Department of Biological Sciences, Purdue University, West Lafayette, IN 47907
NAKAJIMA, YASUKO, Department of Biological Sciences, Purdue University, West Lafayette, IN 47907
NARAHASHI, TOSHIO, Department of Pharmacology, Medical Center, Northwestern University, 303 East Chicago Ave., Chicago, IL 60611
NASATIR, MAIMON, Department of Biology, University of Toledo, Toledo, OH 43606
NELSON, LEONARD, Department of Physiology, Medical College of Ohio, Toledo, OH 43699
NELSON, MARGARET C., Section on Neurobiology and Behavior, Cornell University, Ithaca, NY 14850
NICHOLLS, JOHN G., Department of Neurobiology, Stanford University, Stanford, CA 94305
NICOSIA, SANTO V., Department of OB-GYN, Division of Reproductive Biology, University of Pennsylvania, Philadelphia, PA 19174
NIELSEN, JENNIFER B. K., Merck, Sharp & Dohme Laboratories, Bldg. 50-G, Room 226, Rahway, NJ 07065
NOE, BRYAN D., Department of Anatomy, Emory University, Atlanta, GA 30345
OBAID, ANA LIA, Department of Physiology and Pharmacy, University of Pennsylvania, 4001 Spruce St., Philadelphia, PA 19104
OCHOA, SEVERO, 530 East 72nd St., New York, NY 10021
ODUM, EUGENE, Department of Zoology, University of Georgia, Athens, GA 30701
OERTEL, DONATA, Department of Neurophysiology, University of Wisconsin, 283 Medical Science Bldg., Madison, WI 53706
O'HERRON, JONATHAN, Lazard Freres and Company, 1 Rockefeller Plaza, New York, NY 10020
OLINS, ADA L., University of Tennessee—Oak Ridge, Graduate School of Biomedical Sciences, Oak Ridge National Laboratory, Biology Division, P.O. Box Y, Oak Ridge, TN 37830
OLINS, DONALD E., University of Tennessee—Oak Ridge, Graduate School of Biomedical Sciences, Oak Ridge National Laboratory, Biology Division, P.O. Box Y, Oak Ridge, TN 37830

O'MELIA, ANNE F., George Mason University, 4400 University Drive, Fairfax, VA 22030
OLSON, JOHN M., Institute of Biochemistry, Odense University, Campusvej 55, DK 5230 Odense M, Denmark (resigned 1/84)
OSCHMAN, JAMES L., Marine Biological Laboratory, Woods Hole, MA 02543
PALMER, JOHN D., Department of Zoology, University of Massachusetts, Amherst, MA 01002
PALTI, YORAM, Department of Physiology and Biophysics, Israel Institute of Technology, 12 Haaliya St., BAT-GALIM, POB 9649, Haifa, Israel
PANT, HARISH C., Laboratory of Preclinical Studies, National Institute on Alcohol Abuse and Alcoholism, 12501 Washington Ave., Rockville, MD 20852
PAPPAS, GEORGE D., Department of Anatomy, College of Medicine, University of Illinois, 808 South Wood St., Chicago, IL 60612
PARDEE, ARTHUR B., Department of Pharmacology, Harvard Medical School, Boston, MA 02115
PARDY, ROSEVELT L., School of Life Sciences, University of Nebraska, Lincoln, NE 68588
PARMENTIER, JAMES L., Department of Anesthesiology, Anesthesiology Research Laboratory, 284 Cancer Center/Clinical Bldg., University of South Alabama, Mobile, AL 36688
PASSANO, LEONARD M., Department of Zoology, Birge Hall, University of Wisconsin, Madison, WI 53706
PEARLMAN, ALAN L., Department of Physiology, School of Medicine, Washington University, St. Louis, MO 63110
PEDERSON, THORU, Worcester Foundation for Experimental Biology, Shrewsbury, MA 01545
PERKINS, C. D., 400 Hilltop Terrace, Alexandria, VA 22301
PERSON, PHILIP, Special Dental Research Program, Veterans Administration Hospital, Brooklyn, NY 11219
PETERSON, BRUCE J., Ecosystems Center, Marine Biological Laboratory, Woods Hole, MA 02543
PETHIG, RONALD, School of Electronic Engineering Science, University College of N. Wales, Dean St., Bangor, Gwynedd, LL57 IUT, U. K.
PETTIBONE, MARIAN H., Division of Worms, W-213, Smithsonian Institution, Washington, DC 20560
PFOHL, RONALD J., Department of Zoology, Miami University, Oxford, OH 45056
PIERCE, SIDNEY K., JR., Department of Zoology, University of Maryland, College Park, MD 20740
POLLARD, HARVEY B., NIH, F Building 10, Room 10B17, Bethesda, MD 20205
POLLARD, THOMAS D., Department of Cell Biology and Anatomy, Johns Hopkins University, 725 North Wolfe St., Baltimore, MD 21205
POLLOCK, LELAND W., Department of Zoology, Drew University, Madison, NJ 07940
PORTER, BEVERLY H., 14433 Taos Court, Wheaton, MD 20906
PORTER, KEITH R., 748 Eleventh St., Boulder, CO 80302
POTTER, DAVID, Department of Neurobiology, Harvard Medical School, Boston, MA 02115
POTTER, H. DAVID, P.O. Box 2286, Bloomington, IN 47401
POTTS, WILLIAM T., Department of Biology, University of Lancaster, Lancaster, England, U. K.
POUSSART, DENIS, Department of Electrical Engineering, Universite Laval, Quebec, Canada
PRATT, MELANIE M., Department of Anatomy and Cell Biology, University of Miami School of Medicine (R124), P.O. Box 016960, Miami, FL 33101
PRENDERGAST, ROBERT A., Department of Pathology and Ophthalmology, Johns Hopkins University, Baltimore, MD 21205
PRICE, CARL A., Waksman Institute of Microbiology, Rutgers University, P.O. Box 759, Piscataway, NJ 08854
PRICE, CHRISTOPHER H., Biological Science Center, 2 Cummington St., Boston, MA 02215
PRIOR, DAVID J., Department of Biological Sciences, University of Kentucky, Lexington, KY 40506
PROVASOLI, LUIGI, 474 Whitney Ave., New Haven, CT 06511 (Life Member 12/83)
PRUSCH, ROBERT D., Department of Life Sciences, Gonzaga University, Spokane, WA 99258

PRZYBYLSKI, RONALD J., Case Western Reserve University, Department of Anatomy, Cleveland, OH 44104

PURVES, DALE, Department of Physiology, Washington University School of Medicine, 660 S. Euclid Ave., St. Louis, MO 63110

QUIGLEY JAMES, Department of Microbiology and Immunology Box 44, SUNY Downstate Medical Center, 450 Clarkson Ave., Brooklyn, NY 11203

RABIN, HARVEY, P.O. Box 239, Braddock Heights, MD 21714

RAFF, RUDOLF A., Department of Biology, Indiana University, Bloomington, IN 47405

RAKOWSKI, ROBERT F., Department of Physiology and Biophysics, Washington University, School of Medicine, 660 S. Euclid Ave., St. Louis, MO 63110

RAMON, FIDEL, Dept. de Fisiologia y Biofisca, Centrol de Investigacion y de, Estudius Avanzados del 1pn, Apurtado Postal 14-740, Mexico, D.F. 07000

RANZI, SILVIO, Department of Zoology, University of Milan, Milan, Italy

RATNER, SARAH, Department of Biochemistry, Public Health Research Institute, 455 First Ave., New York, NY 10016

REBHUN, LIONEL I., Department of Biology, Gilmer Hall, University of Virginia, Charlottesville, VA 22901

REDDAN, JOHN R., Department of Biological Sciences, Oakland University, Rochester, MI 48063

REESE, THOMAS S., Marine Biological Laboratory, Woods Hole, MA 02543

REINER, JOHN M., Albany Medical College of Union University, Department of Biochemistry, Albany, NY 12208

REINISCH, CAROL L., Tufts University School of Veterinary Medicine, 203 Harrison Avenue, Boston, MA 02115

REUBEN, JOHN P., Department of Biochemistry, Merck Sharp and Dohme, P.O. Box 2000, Rahway, NJ 07065

REYNOLDS, GEORGE T., Department of Physics, Jadwin Hall, Princeton University, Princeton, NJ 08540

RICE, ROBERT V., Carnegie Mellon Institute, 4400 Fifth Ave., Pittsburgh, PA 15213

RICKLES, FREDERICK R., University of Connecticut, School of Medicine, VA Hospital, Newington, CT 06111

RIPPS, HARRIS, Department of Ophthalmology, New York University School of Medicine, 550 First Ave., New York, NY 10016

ROBERTS, JOHN L., Department of Zoology, University of Massachusetts, Amherst, MA 01002

ROBINSON, DENIS M., High Voltage Engineering Corporation, Burlington, MA 01803

ROCKSTEIN, MORRIS, 335 Fluvia Ave., Miami, FL 33134

RONKIN, RAPHAEL R., 3212 McKinley St., NW, Washington, DC 20015

ROSBASH, MICHAEL, Rosenstiel Center, Department of Biology, Brandeis University, Waltham, MA 02154

ROSE, BIRGIT, Department of Physiology R-430, University of Miami School of Medicine, P.O. Box 016430, Miami, FL 33152

ROSE, S. MERYL, Box 309W, Waquoit, MA 02536

ROSENBAUM, JOEL L., Department of Biology, Kline Biology Tower, Yale University, New Haven, CT 05610

ROSENBERG, PHILIP, School of Pharmacy, Division of Pharmacology, University of Connecticut, Storrs, CT 06268

ROSENBLUTH, JACK, Department of Physiology, New York University School of Medicine, 550 First Ave., New York, NY 10016

ROSENBLUTH, RAJA, 3380 West 5th Ave., Vancouver 8 BC, Canada V6R 1R7

ROSLANSKY, JOHN, Box 208, Woods Hole, MA 02543

ROSLANSKY, PRISCILLA F., Box 208, Woods Hole, MA 02543

ROSS, WILLIAM N., Department of Physiology, New York Medical College, Valhalla, NY 10595

ROTH, JAY S., Division of Biological Sciences, Section of Biochemistry and Biophysics, University of Connecticut, Storrs, CT 06268

ROWLAND, LEWIS P., Neurological Institute, 710 West 168th St., New York, NY 10032

RUDERMAN, JOAN V., Department of Anatomy, Harvard Medical School, Boston, MA 02115

RUSHFORTH, NORMAN B., Case Western Reserve University, Department of Biology, Cleveland, OH 44106

RUSSELL-HUNTER, W. D., Department of Biology, 110 Lyman Hall, Syracuse University, Syracuse, NY 13210

SAFFO, MARY BETH, Department of Biology, Swarthmore College, Swarthmore, PA 19081

SAGER, RUTH, Sidney Farber Cancer Institute, 44 Binney St., Boston, MA 02115

SALAMA, GUY, Department of Physiology, University of Pittsburgh, Pittsburgh, PA 15261

SALMON, EDWARD D., Department of Zoology, University of North Carolina, Chapel Hill, NC 27514

SALZBERG, BRIAN M., Department of Physiology, University of Pennsylvania, 4010 Locust St., Philadelphia, PA 19174

SANDERS, HOWARD, Woods Hole Oceanographic Institution, Woods Hole, MA 02543

SANGER, JEAN M., Department of Anatomy, School of Medicine, University of Pennsylvania, 36th and Hamilton Walk, Philadelphia, PA 19174

SANGER, JOSEPH, Department of Anatomy, School of Medicine, University of Pennsylvania, 36th and Hamilton Walk, Philadelphia, PA 19174

SATO, HIDEMI, Sugashima Marine Biological Laboratory, Nagoya University, Sugashima-cho, Toba-chi, Mie-Ken 517, Japan

SAUNDERS, JOHN, JR., Department of Biological Sciences, SUNY, Albany, NY 12222

SAZ, ARTHUR K., Medical and Dental Schools, Georgetown University, 3900 Reservoir Rd., NW, Washington, DC 20051

SCHACHMAN, HOWARD K., Department of Molecular Biology, University of California, Berkeley, CA 94720

SCHIFF, JEROME A., Institute for Photobiology of Cells and Organelles, Brandeis University, Waltham, MA 02154

SCHLESINGER, R. WALTER, University of Medicine and Dentistry of New Jersey, Department of Microbiology, Rutgers Medical School, P.O. Box 101 Piscataway, NJ 08854 (Life Member 11/83)

SCHMEER, ARLENE C., O.P., Mercene Cancer Research Hospital of Saint Raphael, New Haven, CT 06511

SCHNEIDER, E. GAYLE, University of Nebraska Medical Center, Department of Biochemistry, 42nd and Dewey Ave., Omaha, NE 68105

SCHNEIDERMAN, HOWARD A., Monsanto Company, 800 North Lindberg Blvd., D1W, St. Louis, MO 63166

SCHOPF, THOMAS J. M., Department of Geophysical Sciences, University of Chicago, 5734 South Ellis Ave., Chicago, IL 60637 (deceased 3/84)

SCHOTTE, OSCAR E., Department of Biology, Amherst College, Amherst, MA 01002

SCHUEL, HERBERT, Department of Anatomical Sciences, SUNY, Buffalo, NY 14214

SCHUETZ, ALLEN W., School of Hygiene and Public Health, Johns Hopkins University, Baltimore, MD 21205

SCHWAB, WALTER E., Virginia Polytechnical Institute and State University, Department of Biology, Blacksburg, VA 24601

SCHWARTZ, JAMES H., Center for Neurobiology and Behavior, New York State Psychiatric Institute—Research Annex, 722 W. 168th St., 7th Floor, New York, NY 10032

SCHWARTZ, MARTIN, Department of Biological Sciences, University of Maryland Baltimore County, Catonsville, MD 21228

SCOFIELD, VIRGINIA LEE, Department of Microbiology and Immunology, UCLA School of Medicine, Los Angeles, CA 90024

SCOTT, ALLAN C., 1 Nudd St., Waterville, ME 04901

SCOTT, GEORGE T., 10 Orchard St., Woods Hole, MA 02543 (Life Member 10/83)

SEARS, MARY, P.O. Box 152, Woods Hole, MA 02543

SEGAL, SHELDON J., Population Division, The Rockefeller Foundation, 1133 Avenue of the Americas, New York, NY 10036

SELIGER, HOWARD H., Johns Hopkins University, McCollum-Pratt Institute, Baltimore, MD 21218

SELMAN, KELLY, Department of Anatomy, College of Medicine, University of Florida, Gainesville, FL 32601
SENFT, JOSEPH, 378 Fairview St., Emmaus, PA 18049
SHANKLIN, DOUGLAS R., P.O. Box 1267, Gainesville, FL 32602
SHAPIRO, HERBERT, 6025 North 13th St., Philadelphia, PA 19141
SHAVER, GAIUS R., Ecosystems Center, Marine Biological Laboratory, Woods Hole, MA 02543
SHAVER, JOHN R., Department of Zoology, Michigan State University, E. Lansing, MI 48823
SHEPARD, DAVID C., P.O. Box 44, Woods Hole, MA 02543
SHEPRO, DAVID, Department of Biology, Boston University, 2 Cummington St., Boston, MA 02215
SHERMAN, I. W., Division of Life Sciences, University of California, Riverside, CA 92502
SHILO, MOSHE, Department of Microbiological Chemistry, Hebrew University, Jerusalem, Israel
SHOUKIMAS, JONATHAN J., Marine Biological Laboratory, Woods Hole, MA 02543
SIEGEL, IRWIN, M., Department of Ophthalmology, New York University Medical Center, 550 First Avenue, New York, NY 10016
SIEGELMAN, HAROLD W., Department of Biology, Brookhaven National Laboratory, Upton, NY 11973
SJODIN, RAYMOND A., Department of Biophysics, University of Maryland, Baltimore, MD 21201
SKINNER, DOROTHY M., Oak Ridge National Laboratory, Biology Division, Oak Ridge, TN 37830
SLOBODA, ROGER D., Department of Biological Sciences, Dartmouth College, Hanover, NH 03755
SMITH, HOMER P., Marine Biological Laboratory, Woods Hole, MA 02543
SMITH, MICHAEL A., Colombo Campus, P.O. Box 1490, Colombo 3, Sri Lanka
SMITH, PAUL F., P.O. Box 264, Woods Hole, MA 02543
SMITH, RALPH I., Department of Zoology, University of California, Berkeley, CA 94720
SORENSON, ALBERT L., Albert Einstein College of Medicine, Department of Physiology, 1300 Morris Park Avenue, Bronx, NY 10461
SORENSON, MARTHA M., Depto de Bioquimica-RFRJ, Centro de Ciencias da Saude-I.C.B., Cidade Universitaria-Fundad, Rio de Janeiro, Brasil 21.910
SPECK, WILLIAM T., Case Western Reserve University, Department of Pediatrics, Cleveland, OH 44106
SPECTOR, A., College of Physicians and Surgeons, Columbia University, Black Bldg., Room 1516, New York, NY 10032
SPEER, JOHN W., Marine Biological Laboratory, Woods Hole, MA 02543
SPIEGEL, EVELYN, Department of Biological Sciences, Dartmouth College, Hanover, NH 02755
SPIEGEL, MELVIN, Department of Biological Sciences, Dartmouth College, Hanover, NH 02755
SPRAY, DAVID C., Albert Einstein College of Medicine, Department of Neurosciences, 1300 Morris Park Avenue, Bronx, NY 10461
STEELE, JOHN HYSLOP, Woods Hole Oceanographic Institution, Woods Hole, MA 02543
STEINACHER, ANTOINETTE, Department of Biophysics, The Rockefeller University, New York, NY 10021
STEINBERG, MALCOLM, Department of Biology, Princeton University, Princeton, NJ 08540
STEPHENS, GROVER C., Department of Developmental and Cell Biology, University of California, Irvine, CA 92717
STEPHENS, RAYMOND E., Marine Biological Laboratory, Woods Hole, MA 02543
STETTEN, DEWITT, JR., Senior Scientific Advisor, NIH, Bldg. 16, Room 118, Bethesda, MD 20205
STOKES, DARRELL R., Department of Biology, Emory University, Atlanta, GA 30322
STRACHER, ALFRED, Downstate Medical Center, SUNY, 450 Clarkson Ave., Brookyln, NY 11203
STREHLER, BERNARD L., 2235 25th St., #217, San Pedro, CA 90732
STUART, ANN E., Department of Physiology, Medical Sciences Research Wing 206H, University of North Carolina, Chapel Hill, NC 27514

SUMMERS, WILLIAM C., Huxley College, Western Washington University, Bellingham, WA 98225

SUSSMAN, MAURICE, Department of Life Sciences, University of Pittsburgh, Pittsburgh, PA 15260

SWENSON, RANDOLPHE P., JR., Department of Physiology G-4, University of Pennsylvania, Philadelphia, PA 19174 (resigned 1/84)

SZABO, GEORGE, Harvard School of Dental Medicine, 188 Longwood Avenue, Boston, MA 02115

SZAMIER, R. BRUCE, Harvard Medical School, Berman-Gund Laboratory, Massachusetts Eye and Ear Infirmary, 243 Charles St., Boston, MA 02114

SZENT-GYÖRGYI, ALBERT, Marine Biological Laboratory, Woods Hole, MA 02543

SZENT-GYÖRGYI, ANDREW, Department of Biology, Brandeis University, Waltham, MA 02154

SZENT-GYÖRGYI, EVA SZENTKIRALY, Department of Biology, Brandeis University, Waltham, MA 02154

SZUTS, ETE Z., Laboratory of Sensory Physiology, Marine Biological Laboratory, Woods Hole, MA 02543

TAKASHIMA, SHIRO, Department of Bioengineering, University of Pennsylvania, Philadelphia, PA 19174

TAMM, SIDNEY L., Boston University Marine Program, Marine Biological Laboratory, Woods Hole, MA 02543

TANZER, MARVIN, L., Department of Biochemistry, Box G, Medical School, University of Connecticut, Farmington, CT 06032

TASAKI, ICHIJI, Laboratory of Neurobiology, National Institute of Mental Health, NIH, Bethesda, MD 20205

TAYLOR, DOUGLASS L., Biological Sciences, Mellon Institute, 440 Fifth Avenue, Pittsburgh, PA 15213

TAYLOR, ROBERT E., Laboratory of Biophysics, NINCDS, NIH, Bethesda, MD 20205

TAYLOR, W. ROWLAND, 4800 Atwell Rd., Shady Side, MD 20764

TEAL, JOHN M., Department of Biology, Woods Hole Oceanographic Institution, Woods Hole, MA 02543

TELFER, WILLIAM H., Department of Biology, University of Pennsylvania, Philadelphia, PA 19174

THORNDIKE, W. NICHOLAS, Wellington Management Company, 28 State St., Boston, MA 02109

TRAVIS, D. M., Veterans Administration Medical Center, Fargo, ND 58102

TREISTMAN, STEVEN N., Worcester Foundation for Experimental Biology, Shrewsbury, MA 01545

TRIGG, D. THOMAS, 125 Grove St., Wellesley, MA 02181

TRINKAUS, J. PHILIP, Osborn Zoological Laboratories, Department of Zoology, Yale University, New Haven, CT 06510

TROLL, WALTER, Department of Environmental Medicine, College of Medicine, New York University, New York, NY 10016

TROXLER, ROBERT F., Department of Biochemistry, School of Medicine, Boston University, 80 East Concord St., Boston, MA 02118

TUCKER, EDWARD B., Biology Department, Vassar College, Poughkeepsie, NY 12601

TURNER, RUTH D., Mollusk Department, Museum of Comparative Zoology, Harvard University, Cambridge, MA 02138

TWEEDELL, KENYON S., Department of Biology, University of Notre Dame, Notre Dame, IN 46656

TYTELL, MICHAEL, Department of Anatomy, Bowman Gray School of Medicine, Winston-Salem, NC 27103

URETZ, ROBERT B., Division of Biological Sciences, University of Chicago, 950 East 59th St., Chicago, IL 60637

VALIELA, IVAN, Boston University Marine Program, Marine Biological Laboratory, Woods Hole, MA 02543

VALOIS, JOHN, Marine Biological Laboratory, Woods Hole, MA 02543

VAN HOLDE, KENSAL, Department of Biochemistry and Biophysics, Oregon State University, Corvallis, OR 97331

VILLEE, CLAUDE A., Department of Biological Chemistry, Harvard Medical School, Boston, MA 02115

VINCENT, WALTER S., School of Life and Health Sciences, University of Delaware, Newark, DE 19711

WAINIO, WALTER W., Box 1059 Nelson Labs, Rutgers Biochemistry, Piscataway, NJ 08854

WAKSMAN, BYRON, National Multiple Sclerosis Society, 205 East 42nd St., New York, NY 10017

WALL, BETTY, 9 George St., Woods Hole, MA 02543

WALLACE, ROBIN, A., Department of Anatomy, College of Medicine, University of Florida, Gainesville, FL 32610

WANG, AN, Wang Laboratories, Inc., Bedford Road, Lincoln, MA 01773

WARNER, ROBERT C., Department of Molecular Biology and Biochemistry, University of California, Irvine, CA 92717

WARREN, KENNETH S., The Rockefeller Foundation, 1133 Avenue of the Americas, New York, NY 10036

WARREN, LEONARD, Department of Therapeutic Research, School of Medicine, Anatomy-Chemistry Building, University of Pennsylvania, Philadelphia, PA 19174

WATERMAN, T. H., Yale University, Biology Department, Box 6666, 610 Kline Biology Tower, New Haven, CT 06510

WATSON, STANLEY, Woods Hole Oceanographic Institution, Woods Hole, MA 02543

WEBB, H. MARGUERITE, Marine Biological Laboratory, Woods Hole, MA 02543

WEBER, ANNEMARIE, Department of Biochemistry, School of Medicine, University of Pennsylvania, Philadelphia, PA 19174

WEBSTER, FERRIS, Box 765, Lewes, DE 19958

WEIDNER, EARL, Department of Zoology and Physiology, Louisiana State University, Baton Rouge, LA 70803

WEISS, LEON P., Department of Animal Biology, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, PA 19174

WEISSMANN, GERALD, New York University, 550 First Avenue, New York, NY 10016

WERMAN, ROBERT, Neurobiology Unit, The Hebrew University, Jerusalem, Israel

WESTERFIELD, R. MONTE, The Institute of Neuroscience, University of Oregon, Eugene, OR 37403

WEXLER, NANCY SABIN, 15 Claremont Avenue, Apt. 92, New York, NY 10027

WHITTAKER, J. RICHARD, Boston University Marine Program, Marine Biological Laboratory, Woods Hole, MA 02543

WIERCINSKI, FLOYD J., 21 Glenview Road, Glenview, IL 60025 (Life Member 10/83)

WIGLEY, ROLAND L., 35 Wilson Road, Woods Hole, MA 02543

WILBER, CHARLES G., Department of Zoology, Colorado State University, Fort Collins, CO 80523

WILSON, DARCY B., Department of Pathology, School of Medicine, University of Pennsylvania, Philadelphia, PA 19174

WILSON, EDWARD O., Department of Zoology, Harvard University, Cambridge, MA 02138

WILSON, T. HASTINGS, Department of Physiology, Harvard Medical School, Boston, MA 02115

WILSON, WALTER L., Department of Biology, Oakland University, Rochester, MI 48063

WITKOVSKY, PAUL, Department of Ophthalmology, New York University Medical Center, 550 First Ave., New York, NY 10016

WITTENBERG, JONATHAN B., Department of Physiology and Biochemistry, Albert Einstein College, 1300 Morris Park Ave., New York, NY 10016

WOLF, DON P., Department of OB-GYN, University of Texas Health Sciences Center, 6431 Fannin, Houston, TX 77030

WOLFE, RALPH, Department of Microbiology, 131 Burrill Hall, University of Illinois, Urbana, IL 61801

WOODWELL, GEORGE M., Ecosystems Center, Marine Biological Laboratory, Woods Hole, MA 02543

WORGUL, BASIL V., Department of Ophthalmology, Columbia University, 630 West 168th St., New York, NY 10032

WU, CHAU HSIUNG, Department of Pharmacology, Northwestern University Medical School, 203 E. Chicago Ave., Chicago, IL 60611

WYTTENBACH, CHARLES R., Department of Physiology and Cell Biology, University of Kansas, Lawrence, KS 66045

YEH, JAY Z., Department of Pharmacology, Northwestern University Medical School, 303 E. Chicago Ave., Chicago, IL 60611

YOUNG, RICHARD, Houghton Mifflin Company, One Beacon St., Boston, MA 02108

ZIGMAN, SEYMOUR, School of Medicine and Dentistry, University of Rochester, 260 Crittenden Blvd., Rochester, NY 14620

ZUCKER, ROBERT S., Department of Physiology, University of California, Berkeley, CA 94720

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Putnam, Mr. and Mrs. William A., III
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Robertson, Mrs. C. Stuart
Robertson, Dr. and Mrs. C. W.
Robinson, Dr. and Mrs. Denis M.
Rogers, Mrs. Julian
Root, Mrs. Walter S.
Roslansky, Mr. and Mrs. John
Ross, Dr. Virginia
Roth, Mr. Stephen
Rowe, Mr. and Mrs. William S.
Rubin, Dr. Joseph
Rugh, Mrs. Roberts
Russell, Mr. and Mrs. Henry D.
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Scott, Dr. and Mrs. George T.
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Segal, Dr. and Mrs. Sheldon J.
Senft, Dr. and Mrs. Alfred
Shapiro, Mrs. Harriet S.
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Smith, Mrs. Homer P.
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Snider, Mr. Eliot
Solomon, Dr. and Mrs. A. K.
Specht, Mrs. Heinz
Spiegel, Dr. and Mrs. Melvin
Steele, Mrs. M. Evelyn
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Stetson, Mrs. Thomas J.
Stetten, Dr. DeWitt, Jr.
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Sturtevant, Mrs. A. H.
Swanson, Dr. and Mrs. Carl P.
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Swope, Mrs. Gerard, Jr.
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Waksman, Dr. and Mrs. Byron H.

WARD, DR. ROBERT T.
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ZWILLING, MRS. EDGAR

III. CERTIFICATE OF ORGANIZATION

(On File in the Office of the Secretary of the Commonwealth)

No. 3170

We. Alpheus Hyatt, President, William Stanford Stevens, Treasurer, and William T. Sedgwick, Edward G. Gardiner, Susan Mims and Charles Sedgwick Minot being a majority of the Trustees of the Marine Biological Laboratory in compliance with the requirements of the fourth section of chapter one hundred and fifteen of the Public Statutes do hereby certify that the following is a true copy of the agreement of association to constitute said Corporation, with the names of the subscribers thereto:

We, whose names are hereto subscribed, do, by this agreement, associate ourselves with the intention to constitute a Corporation according to the provisions of the one hundred and fifteenth chapter of the Public Statutes of the Commonwealth of Massachusetts, and the Acts in amendment thereof and in addition thereto.

The name by which the Corporation shall be known is THE MARINE BIOLOGICAL LABORATORY.

The purpose for which the Corporation is constituted is to establish and maintain a laboratory or station for scientific study and investigations, and a school for instruction in biology and natural history.

The place within which the Corporation is established or located is the city of Boston within said Commonwealth.

The amount of its capital stock is none.

In Witness Whereof, we have hereunto set our hands, this twenty seventh day of February in the year eighteen hundred and eighty-eight, Alpheus Hyatt, Samuel Mills, William T. Sedgwick, Edward G. Gardiner, Charles Sedgwick Minot, William G. Farlow, William Stanford Stevens, Anna D. Phillips, Susan Mims, B. H. Van Vleck.

That the first meeting of the subscribers to said agreement was held on the thirteenth day of March in the year eighteen hundred and eighty-eight.

In Witness Whereof, we have hereunto signed our names, this thirteenth day of March in the year eighteen hundred and eighty-eight, Alpheus Hyatt, President, William Stanford Stevens, Treasurer, Edward G. Gardiner, William T. Sedgwick, Susan Mims, Charles Sedgwick Minot.

(Approved on March 20, 1888 as follows:

I hereby certify that it appears upon an examination of the within written certificate and the records of the corporation duly submitted to my inspection, that the requirements of sections one, two and three of chapter one hundred and fifteen, and sections eighteen, twenty and twenty-one of chapter one hundred and six, of the Public Statutes, have been complied with and I hereby approve said certificate this twentieth day of March A.D. eighteen hundred and eighty-eight.

CHARLES ENDICOTT
Commissioner of Corporations)

IV. ARTICLES OF AMENDMENT

(On File in the Office of the Secretary of the Commonwealth)

We. James D. Ebert, President, and David Shepro, Clerk of the Marine Biological Laboratory, located at Woods Hole, Massachusetts 02543, do hereby certify that the following amendment to the Articles of Organization of the Corporation was duly adopted at a meeting held on August 15, 1975, as adjourned to August 29, 1975, by vote of 444 members, being at least two-thirds of its members legally qualified to vote in the meeting of the corporation:

VOTED: That the Certificate of Organization of this corporation be and it hereby is amended by the addition of the following provisions:

"No Officer, Trustee or Corporate Member of the corporation shall be personally liable for the payment or satisfaction of any obligation or liabilities incurred as a result of, or otherwise in connection with, any commitments, agreements, activities or affairs of the corporation.

"Except as otherwise specifically provided by the Bylaws of the corporation, meetings of the Corporate Members of the corporation may be held anywhere in the United States.

"The Trustees of the corporation may make, amend or repeal the Bylaws of the corporation in whole or in part, except with respect to any provisions thereof which shall by law, this Certificate or the bylaws of the corporation, require action by the Corporate Members."

The foregoing amendment will become effective when these articles of amendment are filed in accordance with Chapter 180, Section 7 of the General Laws unless these articles specify, in accordance with the vote adopting the amendment, a later effective date not more than thirty days after such filing, in which event the amendment will become effective on such later date.

In Witness whereof and Under the Penalties of Perjury, we have hereto signed our names this 2nd day of September, in the year 1975, James D. Ebert, President; David Shepro, Clerk.

(Approved on October 24, 1975, as follows:

I hereby approve the within articles of amendment and, the filing fee in the amount of $10 having been paid, said articles are deemed to have been filed with me this 24th day of October, 1975.

PAUL GUZZI
Secretary of the Commonwealth)

V. BYLAWS OF THE CORPORATION OF THE MARINE BIOLOGICAL LABORATORY

(Revised August 11, 1978)

I. (A) The name of the Corporation shall be The Marine Biological Laboratory. The Corporation's purpose shall be to establish and maintain a laboratory or station for scientific study and investigation, and a school for instruction in biology and natural history.

(B) Marine Biological Laboratory admits students without regard to race, color, sex, national and ethnic origin to all the rights, privileges, programs and activities generally accorded or made available to students in its courses. It does not discriminate on the basis of race, color, sex, national and ethnic origin in employment, administration of its educational policies, admissions policies, scholarship and other programs.

II. (A) The members of the Corporation ("Members") shall consist of persons elected by the Board of Trustees, upon such terms and conditions and in accordance with such procedures, not inconsistent with law or these Bylaws, as may be determined by said Board of Trustees. Except as provided below, any Member may vote at any meeting either in person or by proxy executed no more than six months prior to the date of such meeting. Members shall serve until their death or resignation unless earlier removed with or without cause by the affirmative vote of two-thirds of the Trustees then in office. Any member who has attained the age of seventy years or has retired from his home institution shall automatically be designated a Life Member provided he signifies his wish to retain his membership. Life Members shall not have the right to vote and shall not be assessed for dues.

(B) The Associates of the Marine Biological Laboratory shall be an unincorporated group of persons (including associations and corporations) interested in the Laboratory and shall be organized and operated under the general supervision and authority of the Trustees.

III. The officers of the Corporation shall consist of a Chairman of the Board of Trustees, President, Director, Treasurer and Clerk, elected or appointed by the Trustees as set forth in Article IX.

IV. The Annual Meeting of the Members shall be held on the Friday following the Second Tuesday in August in each year at the Laboratory in Woods Hole, Massachusetts, at 9:30 a.m. Subject to the provisions of Article VIII(2), at such meeting the Members shall choose by ballot six Trustees to serve four years, and shall transact such other business as may properly come before the meeting. Special meetings of the Members may be called by the Chairman or Trustees to be held at such time and place as may be designated.

V. Twenty five Members shall constitute a quorum at any meeting. Except as otherwise required by law or these Bylaws, the affirmative vote of a majority of the Members voting in person or by proxy at a meeting attended by a quorum (present in person or by proxy) shall constitute action on behalf of the Members.

VI. (A) Inasmuch as the time and place of the Annual Meeting of Members are fixed by these Bylaws, no notice of the Annual Meeting need be given. Notice of any special meeting of Members, however, shall be given by the Clerk by mailing notice of the time and place and purpose of such meeting, at least 15 days before such meeting, to each Member at his or her address as shown on the records of the Corporation.

(B) Any meeting of the Members may be adjourned to any other time and place by the vote of a majority of those Members present or represented at the meeting, whether or not such Members constitute a quorum. It shall not be necessary to notify any Member of any adjournment.

VII. The Annual Meeting of the Trustees shall be held promptly after the Annual Meeting of the Corporation at the Laboratory in Woods Hole, Massachusetts. Special meetings of the Trustees shall be called by the Chairman, the President, or by any seven Trustees, to be held at such time and place as may be designated. Notice of Trustees' meetings may be given orally, by telephone, telegraph or in writing; and notice given in time to enable the Trustees to attend, or in any case notice sent by mail or telegraph to a Trustee's usual or last known place of residence, at least one week before the meeting shall be sufficient. Notice of a meeting need not be given to any Trustee if a written waiver of notice, executed by him before or after the meeting is filed with the records of the meeting, or if he shall attend the meeting without protesting prior thereto or at its commencement the lack of notice to him.

VIII. (A) There shall be four groups of Trustees:

(1) Trustees (the "Corporate Trustees") elected by the Members according to such procedures, not inconsistent with these Bylaws, as the Trustees shall have determined. Except as provided below, such Trustees shall be divided into four classes of six, one class to be elected each year to serve for a term of four years. Such classes shall be designated by the year of expiration of their respective terms.

(2) Trustees ("Board Trustees") elected by the Trustees then in office according to such procedures, not inconsistent with these Bylaws, as the Trustees shall have determined. Except as provided below, such Board Trustees shall be divided into four classes of three, one class to be elected each year to serve for a term of four years. Such classes shall be designated by the year of expiration of their respective terms. It is contemplated that, unless otherwise determined by the Trustees for good reason, Board Trustees shall be individuals who have not been considered for election as Corporate Trustees.

(3) Trustees ex officio, who shall be the Chairman, the President, the Director, the Treasurer, and the Clerk.

(4) Trustees emeriti who shall include any Member who has attained the age of seventy years (or the age of sixty five and has retired from his home institution) and who has served a full elected term as a regular Trustee, provided he signifies his wish to serve the Laboratory in that capacity. Any Trustee who qualifies for emeritus status shall continue to serve as a regular Trustee until the next Annual Meeting whereupon his office as regular Trustee shall become vacant and be filled by election by the Members or by the Board, as the case may be. The Trustees ex officio and emeriti shall have all the rights of the Trustees, except that Trustees emeriti shall not have the right to vote.

(B) The aggregate number of Corporate Trustees and Board Trustees elected in any year (excluding Trustees elected to fill vacancies which do not result from expiration of a term) shall not exceed nine. The number of Board Trustees so elected shall not exceed three and unless otherwise determined by vote of the Trustees, the number of Corporate Trustees so elected shall not exceed six.

(C) The Trustees and Officers shall hold their respective offices until their successors are chosen in their stead.

(D) Any Trustee may be removed from office at any time with or without cause, by vote of a majority of the Members entitled to vote in the election of Trustees; or for cause, by vote of two-thirds of the Trustees then in office. A Trustee may be removed for cause only if notice of such action shall have been given to all of the Trustees or Members entitled to vote, as the case may be, prior to the meeting at which such action is to be taken and if the Trustee so to be removed shall have been given reasonable notice and opportunity to be heard before the body proposing to remove him.

(E) Any vacancy in the number of Corporate Trustees, however arising, may be filled by the Trustees then in office unless and until filled by the Members at the next Annual Meeting. Any vacancy in the number of Board Trustees may be filled by the Trustees.

(F) A Corporate Trustee or a Board Trustee who has served an initial term of at least 2 years duration shall be eligible for re-election to a second term, but shall be ineligible for re-election to any subsequent term until two years have elapsed after he last served as Trustee.

IX. (A) The Trustees shall have the control and management of the affairs of the Corporation. They shall elect a Chairman of the Board of Trustees who shall be elected annually and shall serve until his successor is selected and qualified and who shall also preside at meetings of the Corporation. They shall elect a President of the Corporation who shall also be the Vice Chairman of the Board of Trustees and Vice Chairman of meetings of the Corporation, and who shall be elected annually and shall serve until his successor is selected and qualified. They shall annually elect a Treasurer who shall serve until his successor is selected and qualified. They shall elect a Clerk (a resident of Massachusetts) who shall serve for a term of 4 years. Eligibility for re-election shall be in accordance with the content of Article VIII (F) as applied to Corporate or Board Trustees. They shall elect Board Trustees as described in Article VIII (B). They shall appoint a Director of the Laboratory for a term not to exceed five years, provided the term shall not exceed one year if the candidate has attained the age of 65 years prior to the date of the appointment. They may choose such other officers and agents as they may think best. They may fix the compensation and define the duties of all the officers and agents of the Corporation and may remove them at any time. They may fill vacancies occurring in any of the offices. The Board of Trustees shall have the power to choose an Executive Committee from their own number as provided in Article X, and to delegate to such Committee such of their own powers as they may deem expedient in addition to those powers conferred by Article X. They shall from time to time elect Members to the Corporation upon such terms and conditions as they shall have determined, not inconsistent with law or these Bylaws.

(B) The Board of Trustees shall also have the power, by vote of a majority of the Trustees then in Office, to elect an Investment Committee and any other committee and, by like vote, to delegate thereto some or all of their powers except those which by law, the Articles of Organization or these Bylaws they are prohibited from delegating. The members of any such committee shall have such tenure and duties as the Trustees shall determine; provided that the Investment Committee, which shall oversee the management of the Corporation's endowment funds and marketable securities, shall include the Chairman of the Board of Trustees, the Treasurer of the Corporation, and the Chairman of the Corporation's Budget Committee, as ex officio members, together with such Trustees as may be required for not less than two-thirds of the Investment Committee to consist of Trustees. Except as otherwise provided by these Bylaws or determined by the Trustees, any such committee may make rules for the conduct of its business; but, unless otherwise provided by the Trustees or in such rules, its business shall be conducted as nearly as possible in the same manner as is provided by these Bylaws for the Trustees.

X. (A) The Executive Committee is hereby designated to consist of not more than ten members, including the ex officio Members (Chairman of the Board of Trustees, President, Director and Treasurer); and six additional Trustees, two of whom shall be elected by the Board of Trustees each year, to serve for a three-year term.

(B) The Chairman of the Board of Trustees shall act as Chairman of the Executive Committee, and the President as Vice Chairman. A majority of the members of the Executive Committee shall constitute a quorum and the affirmative vote of a majority of those voting at any meeting at which a quorum is present shall constitute action on behalf of the Executive Committee. The Executive Committee shall meet at such times and places and upon such notice and appoint such sub-committees as the Committee shall determine.

(C) The Executive Committee shall have and may exercise all the powers of the Board during the intervals between meetings of the Board of Trustees except those powers specifically withheld from time to time by vote of the Board or by law. The Executive Committee may also appoint such committees, including persons who are not Trustees, as it may from time to time approve to make recommendations with respect to matters to be acted upon by the Executive Committee or the Board of Trustees.

(D) The Executive Committee shall keep appropriate minutes of its meetings and its action shall be reported to the Board of Trustees.

(E) The elected Members of the Executive Committee shall constitute as a standing "Committee for the Nomination of Officers," responsible for making nominations, at each Annual Meeting of the Corporation, and of the Board of Trustees, for candidates to fill each office as the respective terms of office expire (Chairman of the Board, President, Director, Treasurer, and Clerk).

XI. A majority of the Trustees, the Executive Committee, or any other committee elected by the Trustees shall constitute a quorum; and a lesser number than a quorum may adjourn any meeting from time to time without further notice. At any meeting of the Trustees, the Executive Committee, or any other committee elected by the Trustees, the vote of a majority of those present, or such different vote as may be specified by law, the Articles of Organization or these Bylaws, shall be sufficient to take any action.

XII. Any action required or permitted to be taken at any meeting of the Trustees, the Executive Committee or any other committee elected by the Trustees as referred to under Article IX may be taken without a meeting if all of the Trustees or members of such committee, as the case may be, consent to the action in writing and such written consents are filed with the records of meetings. The Trustees or members of the Executive Committee or any other committee appointed by the Trustees may also participate in meeting by means of conference telephone, or otherwise take action in such a manner as may from time to time be permitted by law.

XIII. The consent of every Trustee shall be necessary to dissolution of the Marine Biological Laboratory. In case of dissolution, the property shall be disposed of in such a manner and upon such terms as shall be determined by the affirmative vote of two-thirds of the Board of Trustees then in office.

XIV. These Bylaws may be amended by the affirmative vote of the Members at any meeting, provided that notice of the substance of the proposed amendment is stated in the notice of such meeting. As authorized by the Articles of Organization, the Trustees, by a majority of their number then in office, may also make, amend, or repeal these Bylaws, in whole or in part, except with respect to (a) the provisions of these Bylaws governing (i) the removal of Trustees and (ii) the amendment of these Bylaws and (b) any provisions of these Bylaws which by law, the Articles of Organization or these Bylaws, requires action by the Members.

No later than the time of giving notice of the meeting of Members next following the making, amending or repealing by the Trustees of any Bylaw, notice thereof stating the substance of such change shall be given to all Corporation Members entitled to vote on amending the Bylaws.

Any Bylaw adopted by the Trustees may be amended or repealed by the Members entitled to vote on amending the Bylaws.

XV. The account of the Treasurer shall be audited annually by a certified public accountant.

XVI. The Corporation will indemnify every person who is or was a trustee, officer or employee of the Corporation or a person who provides services without compensation to an Employee Benefit Plan maintained by the Corporation, for any liability (including reasonable costs of defense and settlement) arising by reason of any act or omission affecting an Employee Benefit Plan maintained by the Corporation or affecting the participants or beneficiaries of such Plan, including without limitation any damages, civil penalty or excise tax imposed pursuant to the Employee Retirement Income Security Act of 1974; provided, (1) that the Act or omission shall have occurred in the course of the person's service as trustee or officer of the Corporation or within the scope of the employment of an employee of the Corporation

or in connection with a service provided without compensation to an Employee Benefit Plan maintained by the Corporation, (2) that the Act or omission be in good faith as determined by the Corporation (whose determination made in good faith and not arbitrarily or capriciously shall be conclusive), and (3) that the Corporation's obligation hereunder shall be offset to the extent of any otherwise applicable insurance coverage, under a policy maintained by the Corporation or any other person, or other source of indemnification.

VI. REPORT OF THE DIRECTOR

> . . . The future is neither ahead nor behind, on one side or another. Nor is it dark or light. It is contained within ourselves; it is drawn from ourselves; its evil and its good are perpetually within us. The future that we seek from oracles, whether it be war or peace, starvation or plenty, disaster or happiness, is not forward to be come upon. Rather its gestation is now . . .
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> —Loren Eiseley

Introduction

Three changes have led to another one: a change in the form of this Report. First among them was the interest of Trustees in additional meetings, one to be held early in the summer. That interest caused action in February, 1984: scheduling of a meeting for (the 8th and 9th) June, 1984. A particular argument in favor was the need for more time for review of MBL research and scientific policy. The second change followed establishment of the Laboratory's industrial liaison program, whose activities will begin in the summer of 1984. As a part of this program (dubbed the ISP, Interactive Science Program), we have established a database for all MBL investigators and faculty, randomly accessible via their affiliations and research activities. When it is correct and in full use, the database will allow not only periodic rectification of the files, but also inclusion of abstracts on significant research outcomes. It will be printed out in its entirety for the 1984 Decennial Review visitors, and doubtless for many others. Finally, there is a current effort by our Public Information staff to issue new publications on MBL research, some for fund-raising and other external distribution, and some for internal readers.

One result of these actual and proposed changes will be a comprehensive review, in 1984, of all research and training programs at the MBL, and the likely decision to issue a separate report or reports. There is too much of it to fit in the August *Biological Bulletin.* The purposes of this Director's Report will necessarily change. It is, after all, primarily an internal document, read (presumably) by some Corporation members and Trustees; but because of its form of publication and its location amidst legal, statistical, financial, and other membership data, it does not and cannot effectively serve the important purpose of representing MBL science.

Yet the *Biological Bulletin* is the MBL's own journal; and its publication of the Annual Report is widely perceived to be important. That cannot change. Some form of Director's Report must be incorporated; it will continue to be seen by many subscribers, but it will be *read* for the most part by active members of the MBL family. I have chosen, therefore, to try a somewhat different use for it than the yearly history, by Department and by program: to address MBL people quite directly; to avoid discussion of the content of research or teaching, except as these may be immediate issues of policy, saving review of annual accomplishments for other publications; and finally, to address issues of MBL policy and organization with a good deal more frankness than is typical of the alumni magazines produced by college

publications offices. This first trial is concerned with progress and problems. It may entail some risk; but I have never known the truth to cause real trouble when it is truth about something excellent.

Is Progress Necessary?

Readers of about my age may remember a volume by Thurber and White, entitled "Is Sex Necessary?" Its distinguishing feature was the absence of sex. Such is not my intention in choosing the heading for this section. I raise the question about the necessity of progress because it *is* sometimes raised, as such or by implication, within the Corporation. In the time-honored way, the force of the question is blurred by demurrers that argue, not the necessity, but the *definition.* That fools few listeners. Most of us have a pretty good idea of the difference between rest and motion. Most questions attack the idea that it is practical, or necessary, or even in good taste, to move. "Why not," such questions really ask, "stand still?" "After all, things are not so very bad just now; and they were *much* better some time ago."

It is a view with which I can sympathize in principle. I see no progress in electronically-amplified popular music, for example, because it's bad for the Organ of Corti, and most of it isn't music anyway. Nor do I believe that political TV "debates" are debates; that "found objects" are art; that computer games are more than pinball machines; that Transactional Analysis is more effective than reading fiction; or that "computer-literacy" is any kind of literacy.

But the MBL is different. I put the matter bluntly: the MBL of the nineteen-fifties could not *hope* to live in the nineteen-eighties. Most excuses for our financial support in those good old years—and I mean excuses, not the truth (that the MBL was and is the world's most productive Annual Congress of Biology)—have evaporated. With important exceptions such as squid, sea animals, and plants for research can now be shipped to inland locations and used there with some inconvenience. Scientific meetings, and opportunities for travel to them, have multiplied, despite declining grant support. There are several times as many biomedical disciplines and working scientists today as there were in 1950; since the MBL hasn't grown in size to match, the percentage of top people in each field who can be MBL regulars has necessarily declined: we still have a disproportionate share, but we don't any longer have, as we could once claim, nearly all of them. No place has.

The facilities requirements for biomedical science, *e.g.,* equipment and buildings, have grown beyond all expectation. It becomes more difficult and expensive, year by year, to equip MBL laboratories for the advanced work that must be done and taught in them. We are still far ahead of the game, and our courses remain unique in quality; but other institutions are moving aggressively into the business in most MBL fields. Nor can we go back to teaching descriptive, undergraduate-level courses. We couldn't charge the necessary tuition; there would be no grant support for them; and there is strong objection to the idea in many quarters, for it would mean giving up the intellectual frontier we occupy.

It is necessary, in short, for the MBL to understand the times; and to keep, not just abreast, but ahead of them. Change, with minimum dislocation of good, ongoing work; and with minimum hurt to persons, but change measured and according to plan. There is nothing new in this. Old organizations retain support because the public sees their value in simple and immediate terms, not as imponderables or with nostalgia.

The situation might be represented by that universal device for measuring cardiovascular competence: the analytical treadmill. Biomedical science is a treadmill.

For reasons qualitative as well as quantitative, its tilt up is increasing year by year. Doing it, one can't just stop and stand still. Stopping means falling off. That was not true as late as 1960, but it is true today. Therefore *progress is necessary.* We must ask how much progress we are making in the key domains of MBL operations, and what problems there are. Why, for example, too little progress is one area, if that is the judgment; why signs of declining progress elsewhere, even if we are still on track; what are the interactions and contradictions between progress in one domain and the problems of others? These are the kinds of issues I hope to identify below: not to discuss them comprehensively, for that is disallowed by available space and inappropriate for publication with data meant to cover just the year 1983. But I do want to identify some domains and the tensions between progress and problems. I hope that readers among our colleagues may be set to thinking, on their own, about the subject.

Research

The broad measures of our progress in research are seen in many places: Director's Reports of the past few years; statistical data presented to the Corporation and to our donors; the very many publications and achievements, recognized by elections, public honors, and awards, of current members of this community. There are other measures of MBL excellence, a little less direct but nonetheless compelling for those who understand the machinery of science and its support. One example will suffice: the funding of research grant proposals and its relationship to demand for MBL space and accommodations, summer and year-round.

Under prevailing policies of government agencies that support most MBL science, the number of grants of all types awarded each year has been stabilized at what is necessarily a smaller figure that than of earlier years. Although total dollars awarded per grant have risen, purchasing power of the grant dollar has declined. Yet the two direct indicators of success in grant-getting, under the tough peer-reviews that determine it, show the MBL's position, unlike that of many research universities, to be strengthening. Grants to the MBL have been rising, over the past few years, at the remarkable annual rate of 20%. While the number of grants and—more important—the percentage of applicants receiving grants in all fields of MBL interest have stabilized nationally at lower values than in the 1970's, the MBL remains chock-full in summer. The number of requests for year-round research facilities at the MBL has risen or remained level each year since 1978. Despite the absence of any general system of tenure for scientists, people want to make full-time MBL careers; and many are leaders in their fields.

MBL scientists are therefore drawn from a sub-population of the nation's best-qualified biologists: those "approved" applicants who also succeed in getting funded. The dollar squeeze has damaged fine programs elsewhere, and hurt—often unjustly—some distinguished investigators. But the MBL continues to get a full share of the survivors, who must be doing, on the whole, highest-quality science. The squeeze has had, moreover, the interesting (and sometimes painful) consequence that new or younger investigators may get some form of funding preference over the established. Those younger ones, too, want to come to the MBL. A study of recent applicants for MBL laboratory space shows that the funding squeeze, far from reducing the MBL family to a core of the old and established, has in fact led to some turnover and an encouraging number of new and younger applicants.

But there are consequent problems and there will be more. The treadmill will not stop or be lowered to horizontal.

MBL science is the best science; and thus it gets more remote by the month from the microscope, the finger bowls, and the aquaria that could equip an MBL investigator during the early decades. Younger people and new disciplines make demands—legitimate ones—that were unanticipated even ten years ago. They need such unheard-of facilities (for the old MBL) as a proper vivarium for mammals, because, for example, they depend upon immunology and cell culture for the antibodies that are routine tools of cytology and neurobiology. They need larger, more sophisticated, and better-supported facilities for recombinant DNA technology than we have. Computer-assisted imaging and image processing have revolutionized light and electron microscopy. But visiting investigators cannot all bring such gear or the attached technicians with them: it must be here and functional for summer research. As these facilities are established for the year-round programs, they require more space, energy, and trained support staff. Tinker shops are vanishing; skilled instrument makers, shops, and assistants are in rising demand. We must have production-scale mariculture of species essential for research, against the certainty that some of those will become too hard to fish for, and because specimen health and genetics must be defined, as they are not in the wild.

If the MBL is to continue to represent—as has been its mission—the best current thought and techniques in biology, then it will have to spend much *more,* not less, in support of research over the next few years. If it does not, the best science will be done elsewhere: by former MBL investigators who find that they must stay at home in the summers, despite the losses implied, or by those investigators going to other, less comprehensive institutions, equipped, however, for the technical support of their research specialties. Unless we find better methods of recovering the costs of research support and services—including, of course, library and administration—the notable progress made toward placing and keeping the MBL where it belongs on the intellectual ladder of biology will stop. For the reasons mentioned, that means that the sign of progress will be reversed, not merely zeroed.

Education

Kingsley Amis has described (using a very impolite name) the mind-set of certain persons with persistent "views" as a pyramid. The base of this structure I see as at the top, which is composed of many light-weight pieces; each one is an attitude, a slogan, or a canned argument. From top to bottom, the pyramid's pieces get weightier, because the arguments they contain become more personal. At the bottom is the point, and the point is heavy indeed: its argument is *always* personal, and it may be selfish. The supernatural feature of this inverted pyramid is that it is *not* unstable: the disproportionate mass of the point on the ground keeps it stuck upright.

Discourse with persons of such a mind-set can be fun if you know in advance the composition of their point. You can be sure that even if discussion starts at the top of the pyramid, it will end at the bottom, firmly planted in *terra firma.* I think that Mr. Amis has made an important discovery about cognition. We'll return to it shortly.

Judged against the threats to quality science education that have damaged teaching programs in many fine institutions, the MBL has succeeded remarkably well over the past ten years. It has inched forward on the treadmill. Our internationally-known summer courses show nothing like the declines of interest, applicant numbers, and applicant quality that have plagued graduate education elsewhere, to which editorials in *Nature, Science,* and publications of the NSF attest.* The mandated regular turnover

* A case, of more than superficial relevance, in point: Science-Education doctorates. It is well known that the number of doctorates granted in natural science (life sciences, physical sciences, and mathematics)

and updating of MBL course leadership and content goes on, despite pressures from within and without. New courses have been established, *e.g.,* Neural Systems, Parasitism, Microbial Ecology; and they have all succeeded by every strong measure of success.

The January Semester, among off-season programs, has vanished, more or less, because (1) the "free January" has vanished at nearly all of our feeder institutions, and (2) undergraduates are over-committed for tuition payments to their own colleges. But the off-season short course program has grown in quality and recognition. Non-MBL media coverage is good, independent witness. It is now receiving the imaginative input, not only of our own scientists, but also of research and marketing staff from industry, and most recently of clinical specialties (such as neurosurgery) to which MBL strengths are relevant.

Taken *in toto,* the educational program is more than ever, more even than at the founding, inseparable from the rest of the MBL. It is in fact an equal contributor, with independent research, to the values and character of this place. None of this would have been predictable with confidence in, say, 1974. We have made much progress.

Returning now to the mind-set pyramid, it is the main problem inherent in, and working to diminish or reverse, progress. The pyramid's point is different for each category of argument against MBL education as it has evolved. The resulting problem is the same, however, for the effort to sustain orderly operations. Below are some non-imaginary bases (up in the air) and their points (anchored, like grounding rods, in the soil). I admit to coloring them a little for emphasis.

"There are thousands of students out there who could pay realistic tuition fees to study beginning marine invertebrate zoology, marine botany, natural history, if and when you were to return the MBL to its proper and traditional role of teaching those subjects." The point: "Descriptive biology is still important; and that is what I know about. I want a shot at being an MBL faculty member, with all the rights and privileges appertaining thereunto."

"Make 'em pay!" (Students a meaningful tuition fee; Faculty for the research laboratories provided them *gratis*). The point: "I don't see any profit to myself, or my independent research, in the summer courses. Each one is an expensive little world. I don't want to compete with them for facilities and services. I especially don't want my grant money supporting them in luxury."

"We (the course faculty) bring enormous amounts of money to the MBL." The point: "I know the tuition income and the direct-cost budgets for the grants we have; I don't believe the MBL; as I disbelieve my own university, about indirect costs. There is bureaucracy, wasting money, everywhere."

"The provision of services and materials to the courses (independent investigators) should be more (less) centralized." The point: "Mr. _____ (Ms. _____), of the MBL support staff seems unaware that *my* needs are of the highest priority."

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peaked in 1972 and has been declining ever since. But Science-Education doctorates are not the same thing: these are usually granted, after an interval of practical experience, to school and college teachers who have taken some time off for advanced study and the writing of a dissertation. Holders of such degrees are generally headed for Department chairmanships in secondary schools and toward liberal arts colleges. In 1982, the number of doctorates granted had fallen by 60 percent from the peak year, also 1972. The change reflects very complex market conditions, but one of its implications is clear: the demand for instruction in science at the secondary school and junior college level has fallen drastically, even more so than the number of students now enrolling for graduate study in pure science. The 60 percent fall is an underestimate: more than 22 percent of those earning Science-Education doctorates last year were foreigners, most of them holding temporary visas. Note that if, by a miracle, this situation were turned around next year, the half-time for a significant effect upon graduate studies would be of the order of eight years. These data are taken from the NSF's *Mosaic,* Vol. 15, No. 1, 1984.

"Well," the reader may say, if he has followed this far, "what of that? You are describing no more than ordinary human behavior. Why is that a *particular* problem?" My response would be that while it is not a heart-stopper among institutional problems, since we have managed it successfully and will continue to do so, it *is* particular for the MBL's educational program. Perhaps by discussing it, this small one can be disposed of.

The MBL educational program is unique in the world. Among courses providing advanced training in the biomedical sciences, with full-time exposure of students to the most talented faculty and sophisticated instrumentation, those of the MBL summer and short-course programs, taken as a whole, are irreplaceable; an international resource. It would be a violation of the MBL's historic mission to water them down in any way, even upon the questionable hope of tuition income better matched to costs. Program quality has continued to rise, or at least been maintained, despite (1) increased total costs; (2) decreased grant support for direct costs; (3) under-recovery or non-recovery of the indirect costs; and (4) the need for greater expenditures on faculty support, in order to maintain faculty quality in the face of resort-area living expenses.

The strains are felt and they cause vibration at the tops of some pyramids. The real problem causing strain tends to remain unaddressed, except by the few administrators and course directors who work regularly at it. I repeat: without the kind of educational program we have, the MBL would be a different, and a lesser place. The quality of MBL research is in no small measure a result of the combined contributions of independent investigation and the teaching program. That's progress. Entirely new ways of paying for that program *must* be found if the MBL is to go forward, or at least maintain position, on the treadmill. That's the problem.

Finances

I will deal simply with this issue, which is, of those taken up, the most complicated. It is also the least related to those scientific values which we, the Corporation and biologist-Trustees, have been educated to deal with. I can do so because there is no need to provide even a summary of progress; data in this and its predecessor volumes, notably the financial statements and reports of financial officers, summarize it well. The MBL has made good progress in controlling expenses; in keeping its operating budgets decently close to balance over the past few years; and in managing its small but critically important investments. We have the will and the means to keep it thus. But in the very means there is a problem that must grow every year until we find new ones.

I state it as transparently as possible, without doing violence to important details published elsewhere for the trained, or inquiring eye. Let me start with data from a real, but here unnamed, research university. This institution receives Federal agency support, as we do, for research and certain kinds of educational programs (*e.g.*, Training Grants). It is among the distinguished scientific institutions. It has average indirect costs for a mid-sized city without a housing shortage. They are never *fully* recovered, but the recoverable costs are reimbursed by annually-negotiated agency payments figured as a percentage of total direct costs granted. The allocated share of the university's expenses for energy, maintenance, and technical services, payroll management, financial oversight, libraries, clerical and administrative services, becomes the "pooled sponsored research cost." This university has three different pooled cost rates in 1984. One is for the Medical Center; one for the campus that includes the College and most graduate education programs; and one for a large and specialized engineering development laboratory. The approved rates are, respectively, 50%, 70%,

and 53% *of direct costs.* This year, actual recoveries are slightly lower, as a result of a small concession on the 70% component alone.

Let us now make a realistic and very conservative comparison, using the MBL's situation. In a recent year, for which we have full and audited data, the MBL received direct cost payments, on grants and contracts under its full administration, of about $2.97 million. These grants were almost entirely to year-round programs and for some of the courses. Now, the MBL's operating expenses are not solely for work supported by in-house grants. We provide plant, services, housing, library, and so on for the summer population as well. In fact it is much larger than the year-round population, and its grants are made to other institutions. There is no easy way, then, to estimate the direct costs represented by all the grants held by all summer investigators, let alone to allocate correctly the fraction spent during an investigator's months at the MBL. (For example, accounting for and payment of salaries may remain a responsibility of the home institution, but purchases made through the MBL, services such as supplying animals, insuring building safety and security, acquiring equipment, and housing accommodation are all handled here.)

An educated guess as to the direct-cost dollar value of summar research at the MBL can nevertheless be made. I have done so, showing the numbers, at a recent Trustees' meeting. Reducing that last educated guess by about half, I still get an allocated direct cost of about $6 million. The total *effective* direct cost of grant-supported work at the MBL was therefore, in that recent year, more than $9 million.

That year the MBL spent $3.6 million on science support services; which amount was reduced by direct income to departments and from tuition fees to about $2 million. The equivalent of "pooled costs," in other words, amounted to roughly 22% of total direct costs, for research and grant-supported instruction. Total indirect costs actually recovered, as laboratory rental fees, came to $1.6 million, *i.e.,* 18%. This is to be compared with the cost recoveries given earlier for an average research university.

Such data are extractable in proper detail from published financial statements. They have the same outcome every year: the cost of research and advanced education at the MBL is lower than it is at other institutions performing comparable work; the *fraction* recovered by the MBL is *notably* lower than that recovered by peer organizations. For the year in question, our minimum under-recovery was several hundred thousand dollars. I stress "minimum," because some of the support expense was managed by use of private funds. The difference was made up, the budget balanced, more or less, as it has been for the past several years, with investment income and gifts. That is a dangerous course to follow in perpetuity; not only because of increasing uncertainties of private philanthropy, but because it is a waste. It is a waste of the preparation of this and any future MBL Director and Executive Committee to give nearly full time and imagination to soliciting gifts, when they should be given to scientific work and leadership. Worse, it is a waste of privately generated funds, because they could be used for new research initiatives and facilities, rather than to pay routine bills.

There are very few independent laboratories that are as mature, scientifically and culturally, as the MBL. The MBL ought also to be mature in the financial sense that its peer organizations are; *i.e.,* in possession of cost-recovery systems that make at least partial sense in relation to the volume and quality of work done. Such maturity is yet to be attained. In order for it to be attained, we require more than imaginative and expert technical work on the part of financial staff and paid outside advisors: we need clear understanding of the problem by all Trustees and Corporation members, and an unselfish willingness work toward financial systems that are just for the Laboratory as a whole, as well as for its scientists individually.

The symbol of today's situation might be, better than a treadmill, the more

familiar one in Woods Hole of a small cruising sailboat (hull speed: 6.0 knots) heading into maximum current in the Hole (5.8 knots). The two possible outcomes—making it into Vineyard Sound or ending on the rocks east of Devil's Foot—remain a toss-up unless something is done.

The wise skipper starts his engine.

Operating Range and "Ruggedizing"

The foregoing, which contains perhaps too much alternation between the sublime and the ridiculous, does so consciously. At the heart of the message I want to communicate is the issue of operating range for the machine—or organism—that is the MBL. If I have given the impression that the problems discussed are an immediate threat to this Laboratory, then I renounce it. That is not the intention. It is, rather, to suggest that the environment in which this machine is expected to work is different from what it was, and that it will continue to change, within reasonable predictable limits, in the decade ahead.

The environment of concern is mainly external: competing organizations; the pool of funds for science and instruction; the composition and personnel of new disciplines with which we are involved. But the internal environment—the MBL family itself—is also to be considered, although much less urgently than the external one.

Self-regulating machines must be designed to work within environmental limits considerably wider than a few percentage points about the mean. Thus a decent on-board computer for an automobile should be able to withstand, not only normal road vibrations at speed, but an occasional pothole. Its thermal environmental limits should be from well below freezing to at least 115°F. The operating range for computers aboard the space shuttle will be far broader, obviously: otherwise they would fail during the first few seconds of flight.

The design of such machines entails many principles, but three are followed universally and consciously: high component reliability, with some redundancy; "ruggedizing" (awful word!); and simplification.

These principles are often conflicting. The machine's components should, for example, have operating limits at least a little wider than those chosen, with a suitable safety factor, for the complete machine. There should be redundancy, either by duplication of components or via the possibility of circuit-switching, in case of component or connection fails. But what about simplicity? Is it better (or less expensive in the end) to provide a backup chip for each one in service, with all the complications ensuing, or to avoid the complications by choosing a fail-safe chip in the first place, at much greater cost and bulk, but with simplification? There is no formal answer. That's what engineering is about: you find one by a combination of thought and empirical testing.

"Ruggedizing" is even more subtle. Basically, it requires that connections *among* parts, and the housing for all, have wider operating limits even than the components. So you plate circuit boards with noble metals; use carbon fiber and plastics to insulate and hold things together; mount chassis in rubber or something better; and make the external switches moisture- and idiot-proof. But each of those, unless done with thought and care, can end by being more costly than all the components, or by introducing the probability of damage to the components during assembly.

I have tried, in the earlier sections and in what follows, to catalyze objective thinking about the MBL, as though it were a machine or a designable organism; to suggest some of the parameters of its present and future environments, external and internal; and to invite more collaboration among Corporation members and Trustees

in the work of design. For design there must be, if only for retrofitting (to add one more industrial cliche), however excellent the machine may be for its purposes at this time. Nobody in his right mind would judge the MBL a weak machine for its present purposes and time. It is one-of-a-kind, and it's working well. But we are responsible for seeing to it that the MBL works in future time, and that components designed into it are compatible with the future environment. We are responsible for "ruggedizing" it, so that the heat and vibrations, inside and outside, which are a part of the human condition, have no chance of driving it into malfunction.

Governance: The Future Within Us

Academic governance, as the writer of an essay on the subject argued recently at the very start of the piece, is the least (inherently) interesting thing that goes on in a university. It is nevertheless a matter of great practical importance: almost but not quite as important as the teaching and research. And as a matter of fact, governance is of passionate interest among students and faculty. (For the MBL, read students, faculty, and investigators.) Denial of that interest is commonplace; but those elected to serve as, *e.g.,* Trustees are visibly and justifiably proud of it, as are the elected to any exclusive professional body. Those not nominated or elected to such bodies, and yet deserving of it—and there are many such—are hurt by the neglect; they feel pain for a while each year. One doesn't need direct avowals as evidence of pride or hurt: the nature and tone of debate over *causes célèbres* tells the story. Why should the MBL, with its large collection of talented scientists, be different from the rest of Academe?

The interest in governance animates academic debates over process, rights, wrongs, responsibilities, even of style, with an emotional content much higher than that of legislatures and Boards of Directors in the outside world. Thus, the best efforts of TV and the press cannot invest with the academic sense of righteousness a debate on, say, town meeting *versus* mayoral systems for town government. The citizenry have other fish to fry. When the issue is merely selection of one over another local candidate for office, many sensible ones forbear to vote; counting that both a privilege of democracy *and* a vote. I do not necessarily approve: I merely note it.

But, in a faculty meeting, as Corporation members know from home, there is no telling why and on what subject the flash-point may be reached. It may be as weighty a topic as "Are we falling behind the competition in faculty salaries?"; or as seemingly-innocuous as the proposal from Women's Studies for a seminar on "Women in Cosmology." You never can tell. A few pyramids totter on their points, and the fur flies. It's real and it is positive. Whatever the reasons, professors *care* about governance. The great thing is to keep that care channeled, applied toward progress.

Progress has been made here at the MBL. The original Certificate of Organization, dated the 20th March, 1888, was a simple but effective document. Since then there have been numerous revisions and amendments; no single one of them was, in retrospect, other than wise. The last Bylaw revisions of significance were made as recently as 1978. In the formal sense, then, progress in governance—the adjustment of rules and processes to needs of the times—has been steady. The emergent system is unique, just as the institution it serves is unique. But despite care about governance, and progress, problems remain to be solved.

Trustees of academic organizations are usually accomplished persons and are *not* paid members of the community whose well-being is, literally, in their trust. The general idea—one that has worked magnificently for American higher education—is that distinguished outsiders have the wisdom, the technical knowledge, and most important, the *objectivity* to be responsible, ultimately, for institutional policy; to

stand up for it to the faculty as well as to the public. It is sometimes said, in orienting new university Trustees to their tasks, that they have only two real jobs: (1) to select, compensate, and monitor the performance of the Chief Executive Officer; and (2) to "balance the present against the future," in respect of institutional assets, their growth, and their utilization.

These two jobs take time, study, and unselfish devotion. To the extent that my own experience as a university Trustee and as Trustee of research laboratories other than the MBL is normal, the minimum job seems to call for three or four regular meetings per year, each one lasting at least a day and a half. Preparation for a meeting requires a week's evenings of reading and writing, and sometimes many telephone conversations. Every Trustee has visiting committee responsibility, which calls for two to four more working days on location per year, and *very* much more study; in this case of work of the unit or administrative function visited.

Finally, because getting and managing gifts of money is the transcendent concern of scholarly organizations (scholars not normally being heavy earners), and because some Trustees are specialists in money (they have a great deal of it; or can earn it; or manage it professionally; or are close to other people who have a great deal of it), financial policy and action usually originate with the Trustees in consultation with the Chief Executive Officer. Implementation is the job of others: *professional* assistants (*e.g.,* Vice Presidents) to the C.E.O.

In our system at the MBL there is a significant departure from that pattern. It is that twenty-four, at least, of the thirty-six Trustees are—in effect—working members of the faculty; dedicated scholars. They are specifically *not* outsiders. They have a high personal stake in the day-to-day operations of the place. Their views of the work of the Laboratory are technically expert, and not infrequently narrow. It could not be otherwise. MBL Board Trustees, of course, are more like those of other institutions; but the tone and style of the whole Board's activities tend to be set by the majority.

The basic system has worked for nearly a century, which means that no responsible member of the community is going to propose re-writing the Bylaws. It has worked, and it has changed a little, from time to time, to accommodate to external pressures. So far, so good: that is progress. The system was established in 1888 to run ". . . a laboratory or station for scientific study and investigations, and a school for instruction in biology and natural history." The clear understanding was that it would be a *summer* school and station, Woods Hole having been judged uninhabitable the rest of the year. The idea that biologists of the station would also be its owners and policy-managers, under no required constraint of advice from outsiders, was critical to survival of the place during the early decades. The idea was fought over; and happily it triumphed. The job of the resident MBL staff was to take care of the scientists during the summer; and to take care of the plant, such as it was, during the long months when the scientists were back at home.

To be sure, there were indispensable resident managers, the most important of whom must surely be our own Homer Smith: but the clear principle was that high-level policy is made by non-residents, while residents take care of the place, making such lower-level policy as may be needed for the provision of services, the payment of staff (not scientists), and the maintenance of order. A great scientific research hotel, open for the summers. No derogation is implied: it was the world's best of its kind, but still a hotel.

The firm decision to stop being a summer-scientific research hotel was made almost a decade ago. No member of the present Administration, except for Mr. Smith, was here or had any direct role in that decision; hence I can be objective about it, with neither pride in the decision nor the urge to disclaim responsibility for it.

I believe that it was not only the right decision, but *the only possible one.* I have

offered grounds for that belief, in writing, and shall not repeat them here. Suffice it to say that the MBL, functioning as a year-round scientific organization, with extended programs (not just laboratories for rent) and a long-term development plan adopted by the Trustees (in 1979), stepped onto the treadmill of competition. Competition with other free-standing laboratories, with universities and colleges, public and private, and with scholarly organizations of every other kind. Competition for people, recognition, and support.

Many accommodations to that reality have already been made. Quietly, but fairly effectively, procedures have been established for assembling a resident administration (never a mistake-free undertaking, and always traumatic for people already on the scene), which the MBL did not really have until the late 1970's. The job is far from done, but it is being done. Year-round scientific programs have been established. Some have quit but more have flourished, without any reduction of the historic commitment to summer research and teaching. This, too, has not been mistake-free, and trauma has been known to occur at times. Yet the year-round programs exist and do the place honor, as I have shown in earlier Director's Reports. Procedures for regular and searching review of their performance have been established and are being refined. The problem of managing the differences between year-round laboratories and those on location, *i.e.*, summer laboratories, has been met head-on and is being solved, with responsible help of the Research Space Committee and the Executive Committee, neither of which had any such responsibility under the original plan of governance.

We have found several millions of dollars, these past five years, for urgent physical rehabilitation of scientific facilities; and we have spent considerable sums on improvements of housing. Having stepped onto the treadmill, the MBL has managed not to fall off or slip back: we could, today, even tolerate some (unexpected) upward tilt of the bed.

Nevertheless the problems must be faced and solved. Changes in governance of the kind just noted are too much of a patchwork and are not widely understood. Some of them need to be codified. Some need to be extended; some should be reversed. The MBL needs more internal communication; informed guidance; technical management skills; and effective decision-making processes that are not available within the existing system, and which cannot simply be bought or imposed from above. There is no "above." We, the Corporation, are not just the faculty and day-to-day users of the place: we are also the *above.*

No simple handing-out of duties to the existing Board—not even to the twelve Board Trustees—can do the job. Our team of elected officers has insufficient depth in the line positions. They control tens of millions of dollars, in market-value, worth of assets; and an annual volume of ordinary business of the order of ten million. The responsibilities of a thin Administration are great: it is perhaps too personal to describe them as "crushing;" but their undeniable weight is felt by too few persons and there is no practical way, in our system, to spread them over all MBL Trustees, let alone among the more than six hundred non-resident Corporation members. It could be done in 1960; it can't be done today.

I will *not* now discuss solutions to such problems; and we have already rejected Draconian measures, such as rewriting the Bylaws. But I am sure that there are solutions. I hope to be allowed in the next year or two to discuss them with those who care about the MBL. The aim here has been not to suggest specific devices by which we can stay on the treadmill and even gain on it, but rather to argue that such devices are needed, despite the fact that we have not fallen off; and to assert that they exist. They can vary greatly in form. Most versions require no fiddling with the Bylaws. No version requires denial of the MBL's historic missions in science and education,

or allowing program separatism, or the imposition of rules from outside. That was all rejected ninety years ago.

But before specific versions are proposed and argued, there must be real agreement among members of our MBL family that (1) we have made progress, and (2) its continuance requires the possibility of regular changes in governance. It would be the most important of all attractions for the next MBL Director, if he or she is to be the person of quality this great Laboratory deserves, to find that agreements (1) and (2) have been reached; that the way is clear to manage, as management must be done, a successful scientific enterprise of the late Twentieth and early Twenty-first centuries.

Only a Pangloss expects progress without problems. But problems arising out of necessary progress are usually soluble, once their existence is recognized, the alternatives are studied honestly, and their costs and benefits weighed with care. Even nation-states behave that way; once in a great while.

VII. REPORT OF THE TREASURER AND THE CONTROLLER

"Sweet are the uses of adversity . . ."—Shakespeare

Your Treasurer recalls that his father, an incurable optimist, would look upon his son's trying moments as "maturing experiences." This supportive comfort often would be accompanied by a Hoosier proverb, which observes that the same rain that rots the fallen log deepens the roots of the growing tree.

Financially speaking, the MBL had some "maturing experiences" in 1982, culminating in a deficit. The $161,000 excess of expenses over income that year was a modest setback relative to the magnitude of the total budget. Nevertheless, 1982's problem served to strengthen our resolve in 1983 to add momentum to various efforts to increase revenues while intensifying the disciplines of expense management. We are pleased to report that these management actions not only improved the balance between receipts and disbursements, but in fact produced a surplus of $47,000.

To understand 1983's achievement, one must look behind the figures. MBL's commitment to excellence has given the institution a stamina superior to the condition of many struggling not-for-profit organizations. Despite cutbacks in federal funding for research, MBL's laboratories continue to enjoy high levels of occupancy. The implication is that the calibre of the investigators applying for MBL laboratory space is such as to make them relatively more successful in their competition for available funds. Course enrollments, too, have remained high and continue to confirm the excellence of the MBL's educational and training programs. Thus, the historic quality of MBL activities have enabled it to sustain or increase revenues in most all categories.

Specifically, Grants and Restricted Projects increased 12 percent, from last year's $3,476,000 to $3,894,000 in 1983. Laboratory fee income was up 15 percent compared with the previous year. Private gifts increased from $883,000 to $1,199,000, a 33 percent gain.

Progress on the revenue side was matched with achievements in expense control. In the unrestricted budget, operating expenditures in support of research increased a modest $220,000, an increment of less than 6 percent over 1982. A switch from a service bureau's accounting system to an internally operated system, installed on our own recently acquired computer, resulted in faster turn-around on accounting and control reports. One consequence of improved management information was a highly successful effort to reduce Accounts Receivable in the over 90 days category by more than 50 percent. Throughout all operating areas, MBL employees once again demonstrated uncommon resourcefulness in finding ways to control costs while giving strong support to the MBL's purposes.

Two balance sheet categories deserve comment. Fixed Assets are slightly reduced from the 1982 figure, reflecting the fact that the recent pace of new construction declined in 1983 so that fewer new facilities and improvements were put "on the books" last year. This coupled with the normal provision for depreciation, produced an expected decline in the book value of Fixed Assets. The Fund Balance also shows a modest decrease from $164,000 to $149,000, which largely is the result of capitalizing the acquisition of a new computer ($63,600) and renovating Devil's Lane housing ($13,700). Both projects were directly charged to the Fund Balance.

We enter 1984 with more reasons for optimism than we had in 1983's early months. Although the outlook for research funding is no brighter, we feel a bit more comfortable with the MBL's ability to attract funded research to its laboratories. We see encouraging evidence that the arguments employed in our fund raising efforts are persuasive. We have better management tools with which to control expenses and to analyze the effectiveness of our operations. For all of these reasons, we expect to be in a position to give renewed attention to several key objectives for 1984.

Increased participation by the Corporation's membership and Trustees in the development of the MBL's endowment will be one such important objective. Income from invested funds and from trusts benefitting the MBL are important sources of support for our purposes, accounting for 5.6 percent of 1983 income. Nevertheless, our present endowment is not an adequate cushion against financial adversity as its income is entirely consumed by day-to-day operations and therefore unavailable for support to new scientific initiatives or needed improvements to our facilities.

With respect to endowment development, we are most anxious to leverage the challenge grant received from the Andrew W. Mellon Foundation. Successful matching of this grant will add $2.5 million to endowment funds available for library support.

Also in connection with fund raising efforts, two feasibility projects will be completed in 1984. One is studying the physical alternatives and associated costs for the development of a new Marine Resources Center. The second is addressing the feasibility of alternative solutions for additional housing, including the possibility of completing the Swope Center.

Nineteen-eighty-four also will see continued review of MBL's real estate holdings and the policies that have governed the role played by these assets in our financial strategy. Although not generating income, the MBL's undeveloped land in Woods Hole continues to appreciate very significantly because of its scarcity, and holding costs are inconsequential. Our policy to date has been to view this land as an asset to be held in reserve against the possibility of a future financial contingency or to be utilized in some manner consistent with or supportive to the MBL's scientific and educational purposes.

A third important objective for 1984 financial management will be the conclusion of the development and installation of a revised overhead recovery system. Although the need for a new approach has been recognized for some time, the process of reaching internal consensus while negotiating for approvals by cognizant agencies has required careful evaluation of alternatives and thorough preparation of supporting material. We are confident that a more rational approach will increase the MBL's overhead recovery, and of course, that provides a strong incentive to press forward, but at the same time any new system must be fair and equitable to all parties concerned.

Finally, and no less importantly, we will be striving again in 1984 to achieve the income and expense goals of a balanced budget. Put differently, we prefer not to have any "maturing experiences" in 1984.

Respectfully submitted,

John Speer
Controller

Robert Mainer
Treasurer

KEY FINANCIAL INDICATORS 1972–1983

	1972	*1973*	*1974*	*1975*	*1976*	*1977*	*1978*	*1979*	*1980*	*1981*	*1982*	*1983*
UNRESTRICTED												
Revenue	1,142	1,309	1,517	1,663	1,920	2,081	2,502	2,642	3,393	3,552	3,646	4,026
Expenses	1,234	1,396	1,597	1,985	1,814	2,167	2,538	2,611	3,070	3,595	3,807	3,979
Net Inc./Loss	(92)	(87)	(80)	(322)	106	(86)	(36)	31	323	(43)	(161)	47
RESTRICTED												
Grants	602	580	656	952	1,176	1,441	1,756	2,124	2,634	3,041	3,476	3,894
Total Exp. (U + R)	1,836	1,976	2,253	2,937	2,990	3,608	4,294	4,735	5,704	6,636	7,283	7,873
KEY FINANCIAL INDICATORS												
Fund Balance	321	(37)	(176)	(81)	374	627	545	721	808	689	164	149
Invest/Endowment Book Value	3,807	4,123	4,260	5,022	5,398	4,935	5,382	5,836	6,305	6,668	6,825	7,340
Physical Plant	10,009	9,728	9,447	9,129	8,848	8,596	8,362	8,007	8,405	10,065	11,742	11,563
Fund Raising (Private Gifts)	104	124	614	1,019	1,043	997	1,134	1,812	1,760	1,813	883	1,199

Coopers
&Lybrand

certified public accountants

To the Trustees of
Marine Biological Laboratory
Woods Hole, Massachusetts

We have examined the balance sheet of Marine Biological Laboratory as of December 31, 1983 and the related statements of current funds revenue and expenses and changes in fund balances for the year then ended. Our examination was made in accordance with generally accepted auditing standards and, accordingly, included such tests of the accounting records and such other auditing procedures as we considered necessary in the circumstances. We previously examined and reported upon the financial statements of the Laboratory for the year ended December 31, 1982 which condensed statements are presented for comparative purposes only.

In our opinion, the financial statements referred to above present fairly the financial position of Marine Biological Laboratory at December 31, 1983 and its current funds revenue and expenses and the changes in fund balances for the year then ended, in conformity with generally accepted accounting principles, applied on a basis consistent with that of the preceding year.

Coopers & Lybrand

Boston, Massachusetts
June 7, 1984

MARINE BIOLOGICAL LABORATORY

BALANCE SHEETS

December 31, 1983 and 1982

Assets	*1983*	*1982*
Cash and savings deposits	$ 88,099	$ 198,102
Money market securities (Note F)	550,000	665,000
Accounts receivable, net of allowance for uncollectible accounts	209,470	237,859
Receivables due for costs incurred on grants and contracts	500,872	368,958
Other assets	8,031	5,490
Total current assets	1,356,472	1,475,409
Investments, at cost (Note F)	7,340,392	6,825,190
Land, buildings and equipment (Note C)	17,196,770	16,945,601
Less accumulated depreciation	5,633,746	5,203,404
	11,563,024	11,742,197
Total assets	$20,259,888	$20,042,796

Liabilities and Fund Balances	*1983*	*1982*
Accounts payable and accrued expenses	$ 360,758	$ 413,459
Deferred income	62,857	80,089
	423,615	493,548
Current unrestricted fund balance	149,161	163,758
Current restricted fund balance:		
Unexpended grants	86,114	241,409
Unexpended gifts	355,649	358,417
Unexpended income of endowment funds	43,178	29,173
	484,941	628,999
Endowment fund balance:		
Income for unrestricted purposes	1,716,309	1,607,801
Income for restricted purposes	750,434	576,496
	2,466,743	2,184,297
Quasi-endowment fund balance:		
Unrestricted	865,619	816,364
Restricted	2,752,141	2,585,433
	3,617,760	3,401,797
Plant fund balance:		
Unrestricted	11,489,019	11,742,197
Restricted	218,991	163,676
	11,708,010	11,905,873
Retirement fund balance (Note D)	1,409,658	1,264,524
Total liabilities and fund balances	$20,259,888	$20,042,796

The accompanying notes are an integral part of the financial statements.

MARINE BIOLOGICAL LABORATORY

STATEMENTS OF CURRENT FUNDS REVENUE AND EXPENSES

for the years ended December 31, 1983 and 1982

	Unrestricted		*Restricted*		*Total*	
	1983	*1982*	*1983*	*1982*	*1983*	*1982*
Revenue:						
Grant reimbursement of direct costs:						
Instruction			$ 413,797	$ 388,763	$ 413,797	$ 388,763
Research			2,707,011	2,585,101	2,707,011	2,585,101
Recovery of indirect costs related to research and instruction:						
Summer program	$ 428,535	$ 385,104			428,535	385,104
Year-round program	1,342,117	1,149,813			1,342,117	1,149,813
Other	52,298	49,324			52,298	49,324
Instruction	33,331	30,532			33,331	30,532
Tuition	182,217	246,382		12,000	182,217	258,382
Support activities:						
Dormitory	434,686	385,499			434,686	385,499
Dining Hall	247,687	205,902			247,687	205,902
Library	184,325	139,674			184,325	139,674
Biological Bulletin	96,703	99,193			96,703	99,193
Research services	353,051	297,591			353,051	297,591
Marine resources	96,104	85,313			96,104	85,313
Other	(5,786)	925			(5,786)	925
Total support activities	1,406,770	1,214,097			1,406,770	1,214,097
Investment income	232,145	218,910	92,501	371,937	324,646	590,847
Gifts	257,067	231,780	664,190	118,425	921,257	350,205
Miscellaneous revenue	91,559	72,803			91,559	72,803
Total revenue	4,026,039	3,598,745	3,877,499	3,476,226	7,903,538	7,074,971
Expenses:						
Instruction	356,944	309,853	246,323	207,744	603,267	517,597
Research	55,274	29,626	3,371,594	2,803,222	3,426,868	2,832,848
Scholarships and stipends			195,713	319,651	195,713	319,651
Support activities:						
Dormitory	209,176	219,912			209,176	219,912
Dining Hall	235,149	208,775			235,149	208,775
Library	323,660	249,303	76,331	138,446	399,991	387,749
Biological Bulletin	120,822	116,138			120,822	116,138
Research services	543,422	483,407			543,422	483,407
Marine resources	271,224	297,917			271,224	297,917
Administration	959,831	977,607		3,901	959,831	981,508
Plant operation	903,729	866,798	3,516	3,262	907,245	870,060
Total support activities	3,567,013	3,419,857	79,847	145,609	3,646,860	3,565,466
Total expenses	3,979,231	3,759,336	3,893,477	3,476,226	7,872,708	7,235,562
Excess (deficit) of revenue over expenses	$ 46,808	$ (160,591)	$ (15,978)	—	$ 30,830	$ (160,591)

The accompanying notes are an integral part of the financial statements.

MARINE BIOLOGICAL LABORATORY

STATEMENTS OF CHANGES IN FUND BALANCES

for the years ended December 31, 1983 and 1982

		Current Restricted Funds				
	Current Unrestricted Fund	*Unexpended Grants*	*Unexpended Gifts*	*Unexpended Income of Endowment Funds*	*Endowment Funds*	
					Unrestricted	*Restricted*
Balances at beginning of year	$ 163,758	$ 241,409	$358,417	$29,173	$1,607,801	$576,496
Increases:						
Unrestricted current funds revenue	3,536,827					
Grants		4,015,187				
Gifts	257,067	453,952	267,108			138,556
Investment income	232,145	72,242	6,197	30,644		
Realized net gains on sale of investments			2,752		108,508	35,382
Addition to retirement fund						
Tuition						
Decreases:						
Instruction, research and general expenses	(3,979,231)	(3,718,027)	(158,811)	(16,639)		
Indirect costs		(974,640)				
Payments to pensioners						
Depreciation						
Realized net gain (loss) on disposal of fixed assets						
Net change in fund balances before transfers	46,808	(151,286)	117,246	14,005	108,508	173,938
Transfers to (from) funds:						
Acquisition of fixed assets	(85,928)					
Other	24,523	(4,009)	(120,014)			
Balances at end of year	$ 149,161	$ 86,114	$355,649	$43,178	$1,716,309	$750,434

	Quasi-Endowment Funds		*Plants Funds*		*Retirement Funds*	*1983 Total All Funds*	*1982 Total All Funds*
	Unrestricted	*Restricted*	*Unrestricted*	*Restricted*			
Balances at beginning of year	$816,364	$2,585,433	$11,742,197	$163,676	$1,264,524	$19,549,248	$19,176,399
Increases:							
Unrestricted current funds revenue						3,536,827	3,195,431
Grants						4,015,187	3,581,428
Gifts			15,453	67,000		1,199,136	883,197
Investment income		96,859			67,749	505,836	587,379
Realized net gains on sale of investments	49,255	69,849			36,299	302,045	402,640
Addition to retirement fund					87,244	87,244	160,554
Tuition						—	12,000
Decreases:							
Instruction, research and general expenses				(25,455)		(7,898,163)	(7,289,342)
Indirect costs						(974,640)	(773,349)
Payments to pensioners					(46,158)	(46,158)	(26,718)
Depreciation			(430,323)			(430,323)	(360,812)
Realized net gain (loss) on disposal of fixed assets			(9,966)			(9,966)	441
Net change in fund balances before transfers	49,255	166,708	(424,836)	41,545	145,134	287,025	372,849
Transfers to (from) funds:							
Acquisition of fixed assets			192,158	(106,230)		—	—
Other			(20,500)	120,000		—	—
Balances at end of year	$865,619	$2,752,141	$11,489,019	$218,991	$1,409,658	$19,836,273	$19,549,248

The accompanying notes are an integral part of the financial statements.

Notes to Financial Statements

A. *Purpose of the Laboratory:*

The purpose of Marine Biological Laboratory (the "Laboratory") is to establish and maintain a laboratory or station for scientific study and investigations, and a school for instruction in biology and nature history.

B. *Significant Accounting Policies:*

Basis of Presentation—Fund Accounting

In order to ensure observance of limitations and restrictions placed on the use of resources available to the Laboratory, the accounts of the Laboratory are maintained in accordance with the principles of "fund accounting." This is the procedure by which resources are classified into separate funds in accordance with specified activities or objectives. In the accompanying financial statements, funds that have similar characteristics have been combined.

Externally restricted funds may only be utilized in accordance with the purposes established by the donor or grantor of such funds. However, the Laboratory retains full control over the utilization of unrestricted funds. Restricted gifts, grants, and other restricted resources are accounted for in the appropriate restricted funds. Restricted current funds are reported as revenue when expended for current operating or other purposes. Unrestricted revenue is reported as revenue in the unrestricted current fund when earned.

Endowment funds are subject to restrictions requiring that the principal be invested with income available for use by the Laboratory. Quasi-endowment funds have been established by the Laboratory for the same purposes as endowment funds; however, these funds may be expended for various restricted and unrestricted purposes.

Reclassifications

The financial statements for 1983 reflect certain changes in classification of revenue and expenses. Similar reclassifications have been made to amounts previously reported in order to provide consistency of the financial statements. In addition, certain fund balances have been reclassified to appropriately reflect the donors' intentions.

Investments

Investments purchased by the Laboratory are carried at cost. Investments donated to the Laboratory are carried at fair market value at the date of the gift. For determination of gain or loss upon disposal of investments, cost is determined based on the average cost method.

Investment Income and Distribution

The Laboratory follows the accrual basis of accounting except that investment income is recorded on a cash basis. The difference between such basis and the accrual basis does not have a material effect on the determination of investment income earned on a year-to-year basis.

Investment income includes income from the investments of specific funds and from the pooled investment account. Income from the pooled investment account is distributed to the participating funds on the basis of the market value at the beginning of the quarter, adjusted for the cost of any additions or disposals during the quarter.

C. *Land, Buildings and Equipment:*

Following is a summary of the unrestricted plant fund assets:

	1983	*1982*
Land	$ 763,660	$ 720,125
Buildings	14,480,320	14,360,395
Equipment	1,952,790	1,865,081
	17,196,770	16,945,601

Less accumulated depreciation	5,633,746	5,203,404
	$11,563,024	$11,742,197

Depreciation is computed using the straight-line method over estimated useful lives of fixed assets.

D. *Retirement Fund:*

The Laboratory has a noncontributory pension plan for substantially all full-time employees which complies with the requirements of the Employee Retirement Income Security Act of 1974. The actuarially determined pension expenses charged to operations in 1983 and 1982 were $87,244 and $160,554, respectively. The Laboratory's policy is to fund pension costs accrued, as determined under the aggregate level cost method. As of the latest valuation date, based on benefit information at December 31, 1983, the actuarial present values of vested and nonvested benefits, assuming an investment rate of return of 6%, were approximately $1,129,553 and $40,446, respectively. At December 31, 1983, net assets of the plan available for benefits were approximately $1,653,358.

In addition, the Laboratory has a pension plan funded by contributions to the Teachers Insurance and Annuity Association.

E. *Pledges and Grants:*

As of December 31, 1983 and 1982, the following amounts remain to be received on gifts and grants for specific research and instruction programs, and are expected to be received as follows:

	December 31, 1983		*December 31, 1982*	
	Unrestricted	*Restricted*	*Unrestricted*	*Restricted*
1983			$63,000	$ 83,400
1984	$65,000	$293,285		52,000
1985		83,900		
1986		3,965		
	$65,000	$381,150	$63,000	$135,400

In February 1979, the Laboratory initiated the MBL Second Century Fund, a phased effort, to secure $23,000,000 in support of capital rehabilitation, new construction, and endowment. As of December 31, 1983, the Laboratory has received pledges related to this effort of approximately $5,693,358 of which a substantial portion has been collected.

F. *Investments:*

The following is a summary of the cost and market value of investment assets at December 31, 1983 and 1982 and the related investment income and disposition of investment income for the years ended December 31, 1983 and 1982.

	Cost		*Market*		*Investment Income*	
	1983	*1982*	*1983*	*1982*	*1983*	*1982*
Pooled						
U.S. Government securities	$2,352,911	$1,769,146	$2,275,449	$1,743,554	$206,041	$168,171
Corporate fixed income obligations	397,871	711,114	348,713	698,934	32,836	89,394
Common stocks	4,165,295	3,863,773	5,522,238	4,858,263	208,454	239,381
Money market securities	406,766	463,608	406,766	463,608	48,522	38,504
Real estate	17,549	17,549	17,549	17,549	—	—
Total pooled investments	7,340,392	6,825,190	8,570,715	7,781,908	495,853	535,450
Less: custodian fees					(30,282)	(34,138)
					465,571	501,312
Unrestricted Current Fund						
Money market securities	550,000	665,000	550,000	665,000	40,265	86,067
Total investments	$7,890,392	$7,490,190	$9,120,715	$8,446,908	$505,836	$587,379
Disposition of investment income:						
Restricted for current use:						
Utilized in current operations					92,501	371,937
Available for future operations					113,441	(59,342)
Total restricted current and quasi-endowment funds					205,942	312,595
Retirement fund					67,749	55,874
Unrestricted—utilized in current operations					232,145	218,910
					$505,836	$587,379

At December 31, 1983 the following summarizes the participation of the various funds in the investment pool.

Unexpended income of endowment	$ 43,178
Unrestricted endowment	1,716,309
Restricted endowment	682,475
Unrestricted quasi-endowment	846,880
Restricted quasi-endowment	2,788,073
Retirement	1,263,477
	$7,340,392

VIII. REPORT OF THE LIBRARIAN

This past year all the Library collections were brought back to Lillie and reshelved in new locations so that now, after two years of minor confusion, the collection is once more easily accessible to all users. The Rare Books/Archives area on the first floor is a handsome suite of three rooms, one a comfortable Discussion Room, furnished with rugs and Louis Agassiz's large table in the center. The books and archival material are secured in an environmentally controlled area. Fortunately, we now have an Archivist, Ruth Davis, who is organizing and cataloging all Class photographs, correspondence, Meeting minutes, and other archival material. We would be delighted to receive any material of this nature that may be in personal collections of MBL Corporation members.

In March of 1983 we initiated a most comprehensive Survey of the USE made of our entire Journal collection. Cathy Norton of the Library Staff is the Project Coordinator and the funding for this project came from the Rockefeller Foundation. It included purchase of a computer terminal and printer for the Library in order to process all the data received from the Study. *Every* Journal issue that was received from the first of March to the end of December was monitored each time it was used, and all bound journals in the stacks were marked each time they were returned to the shelves. It involved an inordinate amount of record-keeping the results of which will be available in the Fall of 1984, and in the next Annual Report. One most interesting tentative fact; MBL's BIOLOGICAL BULLETIN was referred to over 1,000 times during the ten month survey and was the tenth "most used" journal in the collection of 4,763 separate journal titles.

A second part of the Survey is a USER study which will continue through the summer of 1984. On a number of unannounced days all doors to the Library are locked with the exception of two entrances. All users on that day pass by a desk where they are registered as to institution affiliation. This information will be analyzed at the end of September.

Discussions were held with the National Marine Fisheries Service during the year concerning the incorporation of their library collection with ours in the Main Library. Space has been provided in the Book and Journal stack area for this eventuality.

IX. EDUCATIONAL PROGRAMS

SUMMER

Biology of Parasitism

Course Director

David, John, Harvard School of Public Health/Harvard Medical School

Other faculty, staff, and lecturers

Askanase, Philip, Yale University
Caulfield, John, Harvard Medical School
Chang, Kwang-Poo, Chicago Medical School
Colten, Harvey, Harvard Medical School
Cross, George, The Rockefeller University
David, Roberta, Harvard Medical School
Dessein, Alain, Harvard Medical School
Dwyer, Dennis, NIH
Elsbach, Peter, New York University
Englund, Paul, Johns Hopkins University

FEARON, DOUGLAS, Harvard Medical School
GITLER, CARLOS, Weizmann Institute of Science, Israel
HARN, DONALD, Harvard Medical School
KU, ALBERT, Harvard Medical School
LANDFEAR, SCOTT, Harvard School of Public Health
LODISH, HARVEY, Massachusetts Institute of Technology
MARSDEN, PHILIP, University of Brasilia, Brazil
MCLAFFERTY, MARTHA, Harvard School of Public Health
METZGER, HENRY, NIH
MILLER, LOUIS, NIH
NELSON, GEORGE, Liverpool School of Tropical Medicine, England, U. K.
NUSSENSWEIG, RUTH, New York University
PEREIRA, MIERCIO, Tufts New England Medical Center
PERKINS, MARGARET, The Rockefeller University
PFEFFERKORN, ELMER, Dartmouth College
PIESSENS, WILLY, Harvard University
PRATT, DIANNE, Harvard Medical School
RIFKIN, MARY, The Rockefeller University
ROBERTS, BRYAN, Harvard Medical School
ROSSIGNOL, PHILIPPE, Harvard School of Public Health
SHER, ALAN, NIH
SHERMAN, IRWIN, University of California at Riverside
SPIELMAN, ANDREW, Harvard School of Public Health
SWISTON, LINDA, Mount Holyoke College
WILSON, DARCY, University of Pennsylvania
WIRTH, DYANN, Harvard School of Public Health
WYLER, DAVID, Tufts University Medical School

Students[1]

ANAYA-VELAZQUEZ, LUIS, Center for Research and Advanced Studies of National Polytechnical Institute, Mexico
BHASIN, VIRENDRA, The Rockefeller University
CHAVEZ, LARRY, The University of New Mexico
CSEKO, YARA, Fundacao Oswaldo Cruz, Brazil
DOBBELAERE, DIRK, International Laboratory for Research on Animal Diseases, Kenya
EKAPANYAKUL, GALAYANEE, Mahidol University, Thailand
FAIRFIELD, ALEXANDRA, Cornell University Medical College
FLISSER, ANA, Instituto de Investigaciones Biomedicas, Universidad Nacional Autonoma de Mexico
GOODMAN, HOWARD, Massachusetts General Hospital
KRAKOW, JESSICA, Johns Hopkins University School of Medicine
PERCY, AMY, University of California, Los Angeles
RIVAS-LOPEZ, LUIS, Instituto de Inmunologia y Biologia Microbiana, Spain
ROMERO, GUILLERMO, Instituto de Medicina Tropical "Alexander von Humboldt", Peru
SCHWARZ, RALPH, Deutsche Forschungsgemeinschaft, West Germany
SIBLEY, LAURENCE, Louisiana State University
WYMAN, CLAIRE, Johns Hopkins School of Hygiene and Public Health

EMBRYOLOGY

Course Directors

BRANDHORST, BRUCE, McGill University, Canada
JEFFERY, WILLIAM, University of Texas

[1] All summer students listed completed the formal course program. Asterisk indicates those completing post-course research sessions.

Other faculty, staff, and lecturers

ANGERER, ROBERT, University of Rochester
BATES, WILLIAM, University of Texas
BIGGERS, JOHN, Harvard Medical School
BRODEUR, BONNIE, University of Texas
BRODEUR, RICHARD, University of Texas
BROWER, DANIEL, University of California, Irvine
BROWN, DONALD, Carnegie Institute
BROYLES, ROBERT, University of Oklahoma College of Medicine
CAPCO, DAVID, Massachusetts Institute of Technology
CHAMBERS, EDWARD, University of Miami
CRAIN, WILLIAM, Worcester Foundation
CROSS, NICHOLAS, University of California, Davis
DAVIDSON, ERIC, California Institute of Technology
DESANTIS, ROSARIA, Naples Marine Station, Italy
DRAGO, SALVATORE, University of Toronto
EISEN, ANDREW, University of Pennsylvania
ELINSON, RICHARD, University of Toronto
EMERSON, JULIE, University of California, San Francisco
EPEL, DAVID, Hopkins Marine Station
ETKIN, LARRY, University of Tennessee
GOLDSMITH, MARIAN, University of Rhode Island
GOULD, MEREDITH, University of California at San Diego
GURDON, JOHN, University of Cambridge
HENRY, JONATHAN, University of Texas
HILLE, MERRILL, University of Washington
HOSHI, MOTONORI, Nagoya University, Japan
HUMPHREYS, THOMAS, University of Hawaii
JACOBSON, ALAN, University of Massachusetts Medical School
JAFFE, LAURINDA, University of Connecticut Health Center
JAFFE, LIONEL, Purdue University/Marine Biological Laboratory
KALTHOFF, KLAUS, University of Texas
KLEIN, WILLIAM, Indiana University
MASUI, YOSHIO, University of Toronto
MAXSON, ELLEN, Stanford University
MAXSON, ROBERT, Stanford University
MELTON, DOUGLAS, Harvard University
MILLER, JOHN, University of Calgary, Canada
MOHUN, TIMOTHY, University of Cambridge, U. K.
MOON, RANDY, Caltech
MORROW, LAURA, University of Texas
NELSON, ELLEN, University of Texas
NUCCITELLI, RICHARD, University of California, Davis
NUSSELEIN-VOLHARD, CHRISTIANA, University of Tubingen, West Germany
PEDERSON, THORU, Worcester Foundation for Experimental Biology
PHILLIPS, CAREY, University of California, Berkeley
PHILLIPS, ERIC, University of Texas
PINTO, ANGELO, University of Toronto
RAFF, RUDY, Indiana University
RANKIN, MARY ANN, University of Texas
ROBINSON, KENNETH, University of Connecticut
ROSBASH, MICHAEL, Brandeis University
RUDERMAN, JOAN, Harvard Medical School
SARDET, CHRISTIAN, Villefranche, France
SCHLICTER, LYANNE, University of Connecticut

SCHULTZ, GILBERT, University of Calgary, Canada
SCHULTZ, THOMAS, University of Texas
SCOFIELD, VIRGINIA, Hopkins Marine Station
SHETTLES, BREWER, University of Texas
SPRADLING, AL, Carnegie Institution
SPRAY, DAVID, Albert Einstein College of Medicine
TRINKAUS, J. P., Yale University
VACQUIER, VICTOR, University of California, San Diego
WASSARMAN, PAUL, Harvard Medical School
WEISCHAUS, ERIC, Princeton University
WHITAKER, MICHAEL, University College, London, England, U. K.
WHITTAKER, J. RICHARD, Boston University Marine Program/Marine Biological Laboratory
WILSON, LINDA, University of Texas
WINKLER, MATT, University of Texas
WOODLAND, HUGH, University of Warwick
ZIOMEK, CAROL, Worcester Foundation

Students[1]

BAO, CHENG-YUAN, Case Western Reserve University
*BEACH, REBECCA, University of Connecticut, Storrs
*BEGOVAC, PAUL, University of Florida College of Medicine
*VON BRUNN, ALBRECHT, University of Texas, Austin
*CHUNG, MARGARET, Tufts University School of Medicine
*CONLON, RONALD, McGill University, Canada
*DEAROLF, CHARLES, The Johns Hopkins University
*HALSELL, SUSAN, University of Texas/Patterson Laboratories
*HOWLETT, SARAH, University of Cambridge, England, U. K.
*KLEIN, KAREN, University of Illinois
*LUNDMARK, CATHY, University of California, Berkeley
*LYNCH, EILEEN, The Rockefeller University
*LYONS, GARY, University of Pennsylvania School of Medicine
MARGULES, DEBORAH, University of Michigan
*MILLER, MILL, Tulane University
*NAGY, LISA, University of California, Berkeley
*OSHIRO, DIANNE, University of Virginia
*PEREZ-GRAU, LLUIS, European Molecular Biology Laboratory, West Germany
*ROMANO, CHARLES, University of Massachusetts, Amherst
*SINGER, SUSAN, Rensselaer Polytechnic Institute
*STEVENS, MARY, University of California at Irvine
*SWALLA, BILLIE, University of Iowa
*WANG, ALLAN, University of Hawaii, Manoa

MARINE ECOLOGY

Course Directors

TEAL, JOHN, Woods Hole Oceanographic Institution
VALIELA, IVAN, Boston University Marine Program/Marine Biological Laboratory

Other faculty, staff, and lecturers

ALBERTE, RANDALL, University of Chicago
ANDERSON, DONALD, Woods Hole Oceanographic Institution
BANTA, GARY, Boston University Marine Program/Marine Biological Laboratory
CARON, DAVID, Woods Hole Oceanographic Institution
CONNELL, JOSEPH, University of California, Santa Barbara

DACEY, JOHN, Woods Hole Oceanographic Institution
D'AVANZO, CHARLENE, Hampshire College
DENNISON, WILLIAM, University of Chicago
FRANK, PETER, University of Oregon
GALLAGHER, EUGENE, Marine Biological Laboratory
GIBLIN, ANN, Woods Hole Oceanographic Institution
GILBERT, PATRICIA, Woods Hole Oceanographic Institution
GRASSLE, FREDERICK, Woods Hole Oceanographic Institution
HOBBIE, JOHN, Marine Biological Laboratory
HOWARTH, ROBERT, Marine Biological Laboratory
JANNASCH, HOLGER, Woods Hole Oceanographic Institution
JEFFERIES, ROBERT, University of Toronto, Canada
KOEHL, MIMI, University of California, Berkeley
LAMBERTSEN, R., University of Florida
LEVINTON, JEFFREY, SUNY, New Paltz
MADIN, LAURENCE, Woods Hole Oceanographic Institution
MANN, ROGER, Woods Hole Oceanographic Institution
MARSH, J., University of Guam
NIXON, SCOTT, University of Rhode Island
ODUM, WILLIAM, University of Virginia
PETERSON, SUSAN, Woods Hole Oceanographic Institution
REVELAS, GENE, SUNY, Stony Brook
RIETSMA, CAROL, SUNY, New Paltz
SANDERS, HOWARD, Woods Hole Oceanographic Institution
STOECKER, DIANE, Woods Hole Oceanographic Institution
WELSCHMEYER, N., Harvard University
WOODWELL, GEORGE, Marine Biological Laboratory
WYNES, DAVID, Mount Desert Island Biological Laboratory

Students[1]

*ACKERMAN, JOSEF, SUNY, Stony Brook
BERGGREN, RUTH, Oberlin College
COBLER, SUE, Northeastern University
DONALDSON, JACK, New College of the University of South Florida
*EVANS, ANN, Virginia Institute of Marine Science/College of William and Mary
FEDDELER, WILLIAM, Wayne State University
FREY, JONATHAN, Ohio Wesleyan University
GOLDBERG, SANDRA, Tufts University
HOGUET, NANCY, Barnard College
KESSING, BAILEY, New College of the University of South Florida
LASTA, MARIO, Instituto de Biologia Marina y Pesquera "Alte. Storni," Argentina
*LIEBMAN, MATTHEW, SUNY, Stony Brook
MCCORMICK, DEBORAH, University of Alaska, Anchorage
NEILL, CHRISTOPHER, Louisiana State University
PASCUAL, MARCELA, Instituto de Biologia Marina y Pesquera "Alte. Storni," Argentina
PERKINS, ELEANOR, Marine Biological Laboratory
PROCHAZKA, KARAN, Cambridge, Massachusetts
SLOUGH, DEBRA, Butler University
TAMSE, ARMANDO, Boston University Marine Program/Marine Biological Laboratory
WAGENBACH, GARY, Carleton College

MICROBIAL ECOLOGY

Course Director

HALVORSON, HARLYN, Brandeis University

Other faculty, staff, and lecturers

ALEXANDER, MARTIN, Cornell University
ATWOOD, KIMBALL, Columbia University
AUSICH, RODNEY, Standard Oil of Indiana
BOSTIAN, KEITH, Brown University
CASTENHOLZ, RICHARD, University of Oregon
CAVANAUGH, COLLEEN, Harvard University
CRONIN, JOHN, University of Illinois
DAVIS, BERNARD, Harvard University School of Medicine
DWORKIN, MARTIN, University of Minnesota
GRAY, T. R. G., University of Essex, England, U. K.
GREENBERG, E. PETER, Cornell University
HANSEN, RICHARD, Gray Freshwater Biological Institute
HOBBIE, JOHN, Marine Biological Laboratory
HUMPHREY, ARTHUR, Air Products and Chemicals, Inc.
JANNASCH, HOLGER, Woods Hole Oceanographic Institution
KEYNAN, ALEXANDER, Hebrew University, Jerusalem, Israel
KORNBERG, HANS, Cambridge University, England, U. K.
LEADBETTER, EDWARD, University of Connecticut, Storrs
MORTENSON, LEONARD, Exxon Research and Engineering Company
ORNSTON, L. N., Yale University
PIERSON, BEVERLY, University of Puget Sound
POINDEXTER, JEANNE, Public Health Research Institute, New York, NY
RICH, ALEX, Massachusetts Institute of Technology
ROMESSER, JAMES, Dupont Corporation
RUBY, EDWARD, University of Southern California
SCHAECHTER, M., Tufts University
SHILO, MOSHE, Life Science Institute of Hebrew University, Jerusalem, Israel
VINCENT, WALTER, University of Delaware
WATERBURY, JOHN, Woods Hole Oceanographic Institution
WOSE, CARL, University of Illinois
WOLFE, RALPH, University of Illinois

Students[1]

ACKERMAN, EUGENE, University of Arkansas
BOYER, JOSEPH, Virginia Institute of Marine Science/College of William and Mary
DECHO, ALAN, Louisiana State University
ESCHER, ANDREAS, Montana State University College of Engineering
HARTEN, JAMES, Vanderbilt University
HARTZELL, PATRICIA, University of Illinois
JOUPER, ASA, Gothenberg University, Sweden
KIEFT, THOMAS, New Mexico Highlands University
MALMCRONA-FRIBERG, KARIN, Gothenberg University, Sweden
MUEHLSTEIN, LISA, Wright State University
NOLL, KENNETH, University of Illinois
ROBERTSON, CHARLES, Skidaway Institute of Oceanography
SANTORO, NICHOLAS, Ohio State University
SKLADANY, GEORGE, Clemson University
SUTHERLAND, DALE, Creighton University
SUTTON, WILLIAM, Auburn University
WRENN, BRIAN, University of Miami Rosenstiel School of Marine and Atmospheric Sciences

Neural Systems and Behavior

Course Directors

Hoy, Ronald, Cornell University
Macagno, Eduardo, Columbia University

Other faculty, staff, and lecturers

Alkon, Daniel, NINCDS/Marine Biological Laboratory
Bennet-Clark, Henry, Oxford University, England, U. K.
Calabrese, Ronald, Harvard University
Carew, Thomas, Yale University
Chalfie, Martin, Columbia University
Flanagan, Thomas, Cold Spring Harbor Laboratory
Getting, Peter, University of Iowa
Gould, James, Princeton University
Grinvald, Amiram, Weizmann Institute, Israel
Haerter, Ursula, Columbia University
Harris-Warrick, Ronald, Cornell University
Hopkins, Carl, Cornell University
Kandel, Eric, Columbia College of Physicians and Surgeons
Kelley, Darcy, Columbia University
Kravitz, Edward, Harvard Medical School
Kristan, William, University of California, San Diego
Lent, Charles, Brown University
Marder, Eve, Brandeis University
Marler, Peter, The Rockefeller University
McVey, Margaret, The Rockefeller University
Menzel, Randolf, Free University of Berlin, West Germany
Murphey, Rodney, SUNY, Albany
Musacchio, Michele, Columbia University
Nelson, Margaret, Cornell University
Nicholls, John, Stanford University School of Medicine/Biocenter, Basel, Switzerland
Rehder, Vincent, Institut fur Neurobiologie, West Germany
Sahley, Christine, Yale University
Sassoon, David, Columbia University
Segil, Neil, Columbia University
Silver, Rae, Columbia University
Stewart, Randy, Columbia University
Wiesel, Torsten, Rockefeller University
Zipser, Birgit, Cold Spring Harbor Laboratory

Students[1]

*Baptista, Carlos, University of Lisbon, Portugal
Beason, Robert, SUNY, Geneseo
*Bird, Edythe, Yale University
Borst, Alexander, Institut fur Genetik und Mikrobiologie, West Germany
Coon, Steven, University of Maryland
*Delaney, Kerry, Princeton University
*Gaide, Michael, Free University of Berlin, West Germany
Gilliam, David, University of Colorado
Kuterbach, Deborah, SUNY, Stony Brook
Lander, Eric, Harvard University
Lannoo, Michael, Dalhousie University, Nova Scotia, Canada
Lesk, Mark, The Weizmann Institute of Science, Israel

LI, CHRISTINE, Harvard University
NOBLE, MICHAEL, St. Mary's Hospital Medical School, University of London, U. K.
RISKA, DIANE, University of California, Los Angeles
SIMS, STEPHEN, Columbia University
SMITH, JEFFREY, Harvard School of Public Health
STEVENS, CRAIG, University of Illinois, Chicago
WEINER, GEORGE, Harvard University
*WORDEN, MARY, University of Chicago

NEUROBIOLOGY

Course Directors

HILDEBRAND, JOHN, Columbia University
REESE, THOMAS, NINCDS/NIH/Marine Biological Laboratory

Other faculty, staff, and lecturers

ARMSTRONG, CLAY, University of Pennsylvania
BATTELLE, BARBARA, National Eye Institute, NIH
BENTLEY, DAVID, University of California at Berkeley
BRAY, DENNIS
BURD, GAIL, The Rockefeller University
CONSTANTINE-PATTON, MARTHA, Princeton University
CHRISTAKIS, NICHOLAS, Yale University
DUNLAP, KATHLEEN, Tufts University Medical School
EVANS, PETER, University of Cambridge, England, U. K.
FISCHBACH, GERALD, Washington University School of Medicine
FURSHPAN, EDWIN, Harvard Medical School
GOY, MICHAEL, Harvard Medical School
HALL, LINDA, Albert Einstein College of Medicine
HUTTNER, SUSANNE, University of California, Los Angeles
JACOBSON, MARCUS, University of Utah College of Medicine
KACHAR, BECHARA, NINCDS, NIH
KENT, KARLA, Columbia University
KINNANE, JANET, Marine Biological Laboratory
LAFRATTA, JAMES, Harvard Medical School
LANDIS, DENNIS, Massachusetts General Hospital
LANDIS, STORY, Harvard Medical School
MARLER, JENNI, McGill University, Canada
MATSUMOTO, STEVEN, Harvard Medical School
MICHAUD, JAYNE, Marine Biological Laboratory
NICAISE, GHISLAIN, Universite Claude Bernard, France
NICAISE, MARI, Universite Claude Bernard, France
NISHI, RAE, Harvard Medical School
O'CONNELL, MAUREEN, NINCDS, NIH
O'LAGUE, PAUL, University of California, Los Angeles
PATTERSON, PAUL, Harvard Medical School
POTTER, DAVID, Harvard Medical School
PURVES, DALE, Washington University School of Medicine
RAVIOLA, ELIO, Harvard Medical School
REESE, BARBARA, NINCDS, NIH
ROSENBLUTH, JACK, New York University School of Medicine
SALZBERG, BRIAN, University of Pennsylvania
SCHNAPP, BRUCE, NINCDS, NIH/Marine Biological Laboratory
SHOTTON, DAVID, University of Oxford, England, U. K.
STEVENS, JOHN, Toronto Western Hospital, Canada

THOENEN, HANS, Max Planck Institute for Psychiatry, West Germany
WALROND, JOHN, NINCDS, NIH/Marine Biological Laboratory
WIESEL, TORSTEN, Harvard Medical School
WILLARD, MARK, Washington University School of Medicine
ZIGMOND, RICHARD, Harvard Medical School

Students[1]

BAETGE, GREG, Columbia University
BOARDMAN, IAN, University of Pennsylvania
BODMER, ROLF, Friedrich Miescher Institut, Switzerland
DE SANTIS, AMEDEO, Stazione Zoologica de Napoli, Italy
GOODMAN, LINDA, The Albert Einstein College of Medicine
MARSH, TERRY, National Jewish Hospital and Research Center
NEW, JOHN, Wesleyan University
PEINADO, ALEJANDRO, Columbia University
RUANO-ARROYO, GUALBERTO, Yale University School of Medicine
SCHRANK, ETHAN, University of North Carolina at Chapel Hill
WITTEN, JANE, University of Chicago

PHYSIOLOGY

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ROSENBAUM, JOEL, Yale University

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ACKERS, GARY, Johns Hopkins University
ALLEWELL, NORMA, Wesleyan University
ALTMAN, SIDNEY, Yale University
AUSUBEL, FRED, Harvard University
BALTIMORE, DAVID, Massachusetts Institute of Technology
BEGG, DAVID, Harvard Medical School
BLOODGOOD, ROBERT, University of Virginia
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BRINKLEY, WILLIAM, Baylon University Medical School
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CENTONZE, VICKY, Dartmouth College
CHISHOLM, REX, Massachusetts Institute of Technology
CONDEELIS, JOHN, Albert Einstein College of Medicine
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GOLDMAN, ROBERT, Northwestern University Medical School
GREENE, KATHLEEN, Northwestern University Medical School
HEREFORD, LYNNA, Brandeis University
HOTANI, Q-CHAN, University Kyoto, Japan
HUNT, TIMOTHY, Cambridge University, England, U. K.
INOUÉ, SHINYA, Marine Biological Laboratory
JOHNSON, ROSS, University of Minnesota
JONES, JONATHAN, Northwestern University Medical School
KAUMEYER, JOHN, University of Pennsylvania
KILMARTIN, JOHN, Medical Research Council, England, U. K.
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KUMAR, AJIT, George Washington University Medical Center
KURIYAMA, RYOKO, University of Wisconsin

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MITCHELL, DAVID, Yale University
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MURRAY, ANDREW, Harvard University
PANT, HARISH, National Institute on Alcohol Abuse and Alcoholism
REID, MARTHA, Earlham College
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ROSENTHAL, ERIC, Harvard University Medical School
RUSHFORTH, ALICE, Earlham College
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SILFLOW, CAROLYN, University of Minnesota
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SLUDER, KIP, Worcester Foundation
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VALLEE, RICHARD, Worcester Foundation
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ZACKROFF, ROBERT, Northwestern University Medical School

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JANUARY

BEHAVIOR

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KAMIL, AL, University of Massachusetts
KREITHEN, MEL, University of Pittsburgh
KROODSMA, DONALD, University of Massachusetts
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MOLLER, PETER, American Museum of Natural History
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PAYNE, ROGER, Lincoln, Massachusetts
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TAYAK, PETER, Woods Hole Oceanographic Institution
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TROTT, THOMAS, Boston University Marine Program/Marine Biological Laboratory
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HUTCHINSON, LINDA, Boston University
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COMPARATIVE PATHOLOGY OF MARINE INVERTEBRATES

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REINISCH, CAROL, Tufts University School of Veterinary Medicine

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FARLEY, AUSTIN, Oxford Marine Research Laboratory
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SINDERMAN, CARL, National Marine Fisheries Service
SPARKS, ALFRED, University of Washington School of Fisheries
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STEWART, JAMES, Fisheries Research Branch, Nova Scotia, Canada
STRANDBERG, JOHN, Johns Hopkins University School of Medicine
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FORE, STEPHANIE, St. Andrews Presbyterian College
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KANUNGO, KALPATARU, Western Connecticut State University
LIMA, GAIL, Tufts University
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SHORT COURSES

ANALYTICAL AND QUANTITATIVE LIGHT MICROSCOPY IN BIOLOGY, MEDICINE, AND MATERIALS SCIENCES

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WOODWARD, BERTHA, Marine Biological Laboratory

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PAGLIARO, LEONARD, Wesleyan University
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SAFT, MALLORY, University of Health Sciences/The Chicago Medical School
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SIZTO, NING-LEUNG, Yale University School of Medicine
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ENNEKING, KITTY, Hacker Instruments
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RICHARDS, ANN, Burroughs Wellcome Company
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MARICULTURE: CULTURE OF MARINE INVERTEBRATES FOR RESEARCH PURPOSES

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BOWER, CAROL, Institute for Aquarium Studies
CAPO, THOMAS, Marine Biological Laboratory
CAPUZZO, JUDITH, Woods Hole Oceanographic Institution
DOYLE, ROGER, Dalhousie University
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GARIBALDI, LOUIS, New York Aquarium
GUILLARD, ROBERT, Bigelow Laboratories
HANLON, ROGER, Marine Biomedical Institute
HARRIGAN, JUNE, Marine Biological Laboratory
HUGHES, JOHN, Massachusetts State Lobster Hatchery
KERR, LOUIS, Marine Biological Laboratory
LEIBOVITZ, LOUIS, Marine Biological Laboratory
MANN, ROGER, Woods Hole Oceanographic Institution
MARCUS, NANCY, Woods Hole Oceanographic Institution
SPOTTE, STEPHEN, Mystic Marinelife Aquarium
SULKIN, STEPHEN, Horn Point Laboratory
TURNER, DAVID, Institute for Aquarium Studies

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AL-YAMANI, FAIZA, University of Miami
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CASTELLI, MAURIZIO, Virginia Institute of Marine Science
CHECA, MIGUEL, Aquamundo, Peru
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LANDY, RONALD, New York State College of Veterinary Medicine/Cornell University

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LUBZENS, ESTHER, Israel Oceanographic and Limnological Research Ltd., Israel
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MLADENOV, PHILIP, Mount Allison University
NADEAU, LLOYD, Marine Biological Laboratory
RUANO, FRANCISCO, Northeast Fisheries Center
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SHEPHERD, GORDON, Yale University School of Medicine
WELSH, MICHAEL, University of Iowa Hospitals

Protein Analysis by Polyacrylamide Gel Electrophoresis

Course Directors

Stephens, Raymond, Boston University School of Medicine/Marine Biological Laboratory
Zweidler, Alfred, The Institute for Cancer Research

Other faculty, staff, and lecturers

Good, Michael, Marine Biological Laboratory
Kerr, Louis, Marine Biological Laboratory
Masure, H. Robert, Boston University School of Medicine

Students

Adams, Susan, VA Medical Center, Kansas City, Missouri
Brower, Danny, University of California, Irvine
Campenot, Robert, Cornell University
Chepko, Gloria, Albert Einstein School of Medicine
Chou, Ta-Hsu, Michigan Cancer Foundation
Donady, J. James, Wesleyan University
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Ganz, Peter, Brigham and Women's Hospital
Hayhome, Barbara, University of Nebraska, Omaha
Kazura, James, University Hospitals
Koopmans, Henry, Columbia University
Kuhns, William, University of North Carolina
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McGrath, Ann, VA Hospital, San Francisco, California
Pearson, James, The Upjohn Company
Ripps, Harris, New York University School of Medicine
Roesijadi, Guri, Battelle Marine Research Laboratory
Troncoso, Juan, Johns Hopkins University
Walter, Anne, NIH—National Heart, Lung and Blood Institute

Small Computers in Biomedical Research

Course Director

Palmer, Larry, University of Pennsylvania School of Medicine

Other faculty, staff, and lecturers

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Jones, Judson, University of Pennsylvania School of Medicine
Peachey, Lee, University of Pennsylvania

Students

Bruce, Richard, Highlands Biological Station
Chen, Lee, University of California
Cheng, Toni, Marine Biological Laboratory
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Hester, Kelly, Texas A&M University College of Medicine
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Kusumi, Akihiro, Princeton University
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Sheff, Michael, The Miriam Hospital
Sundell, Cynthia, University of Pennsylvania

X. RESEARCH AND TRAINING PROGRAMS

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ALBERTE, RANDALL S., University of Chicago, Barnes Laboratory
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ALLEN, ROBERT D., Dartmouth College
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ARNOLD, JOHN M., Kewalo Marine Laboratory, Pacific Biomedical Research Center
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BORGESE, THOMAS A., Lehman College, City University of New York
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COHEN, WILLIAM D., Hunter College
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GILBERT, DANIEL L., NIH
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HAIMO, LEAH T., University of California, Riverside
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HIGHSTEIN, STEPHEN, Albert Einstein College of Medicine

HUMPHREYS, TOM, University of Hawaii
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INGOGLIA, NICHOLAS A., UMDNJ—New Jersey Medical School
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LANDOWNE, DAVID, University of Miami
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LIPICKY, JOHN, Food & Drug Administration
LLINAS, RODOLFO, New York University Medical Center
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CARRIERE, RITA, SUNY, Downstate Medical Center
CHATTERJEE, DEB K., University of Illinois
CHILD, FRANK M., Trinity College
CLARK, ARNOLD, University of Delaware
COHEN, SEYMOUR S., SUNY, Stony Brook
COLLIER, JACK R., Brooklyn College
COLLIER, MARJORIE M., Saint Peter's College
COUCH, ERNEST F., Texas Christian University
COWLING, VINCENT F., SUNY, Albany
CROWLEY, WILLIAM F., Massachusetts General Hospital
DESSNER, DANIEL A., Kenyon College
DETTBARN, WOLF-D., Vanderbilt University Medical Center
DUNCAN, THOMAS, Marine Biological Laboratory
DUNN, STEPHEN, Harvard University
EBERT, JAMES, Carnegie Institute of Washington
EDDS, LOUISE L., Ohio University
EDER, HOWARD A., Albert Einstein College of Medicine
ELLNER, JERROLD, University Hospitals, Cleveland, Ohio
EPEL, DAVID, Stanford University

FEINGOLD, DAVID S., New England Medical Hospital
FISHER, SAUL, New York University School of Medicine
FLAHERTY, CLAIRE V., Fairleigh Dickenson University
FLISSER, ANNA, Instituto de Investigaciones Biomedicas, Mexico
FREINKEL, NORBET, Northwestern University Medical School
FRIEDLER, GLADYS, Boston University School of Medicine
FROST, JOHN KINGSBURY, John Hopkins School of Medicine
GABRIEL, MORDECAI L., Brooklyn College
GERMAN, JAMES L., The New York Blood Center
GOLDSTEIN, LESTER, University of Kentucky
GOLDSTEIN, MOISE H., John Hopkins University
GOODE, DENNIS, University of Maryland
GRANT, PHILIP, University of Oregon
GROSCH, DANIEL S., North Carolina State University
GROSSMAN, ALBERT, New York University Medical Center
GUTTENPLAN, JOSEPH B., New York University College of Dentistry
HAN, JON, Kenyon College
HARDING, CLIFFORD V., Kresge Eye Institute of Wayne State University
HAUBRICH, ROBERT R., Denison University
HERNANDEZ-NICAISE, MARI-LUZ, Universite Claude Bernand, France
HUBBARD, RUTH, Harvard University
HUFNAGEL, LINDA, University of Rhode Island
ILAN, JOSEPH D., Case Western Reserve University School of Medicine
INOUÉ, SADAYUKI, McGill University
INOUÉ, SHINYA, Marine Biological Laboratory/University of Pennsylvania
ISSENBERG, IRVIN, Oregon State University
JOHNSON, MICHAEL, Harvard Medical School
JONES, MEGAN, Harvard University
KANE, ROBERT E., University of Hawaii
KALTENBACH, JANE C., Mount Holyoke College
KAWAI, MASATAKA, Columbia University
KIMANI, ROBINSON GAUCHUHI, Clinical Research Centre, Nairobi, Kenya
KIRSCHENBAUM, DONALD M., College of Medicine, SUNY
KOSOWER, EDWARD M., Tel-Aviv University, Israel
KRAVITZ, EDWARD A., Harvard Medical School
LADERMAN, AIMLEE D., Smithsonian Institution
LAZAROW, PAUL B., The Rockefeller University
LEE, JOHN J., City College of New York
LEIGHTON, JOSEPH, The Medical College of Pennsylvania
LEVINE, RACHMIEL, City of Hope Medical Center, California
LEVINE, WALTER G., Albert Einstein College of Medicine
LEVITZ, MORTIMER, New York University Medical Center
LO, WOO-KUEN, Kresge Eye Institute of Wayne State University
LORAND, LASZLO, Northwestern University
MAIENSCHEIN, JANE, Arizona State University
MARINE RESEARCH, Falmouth, Massachusetts
MASER, MORTON, Woods Hole, Massachusetts
MAUTNER, HENRY G., Tufts University School of Medicine
MAUZERALL, DAVID, The Rockefeller University
MEINERTZHAGEN, I. A., Dalhousie University, Nova Scotia
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MILLER, DANIEL G., PMI-Strang Clinic
MITCHELL, JAMES B., Moravian College
MIZELL, MERLE, Tulane University
MONSANTO COMPANY, St. Louis, Missouri
MONROY, ALBERTO, Stazione Zoologica, Napoli, Italy
MOORE, JOHN W., Duke University

MORSE, STEPHEN, Rutgers University
MUSICK, JIM, Ultra Pure Laboratories, Salt Lake City
O'RAND, ANGELA, Duke University
OSCHMAN, JAMES, Woods Hole, Massachusetts
ORME-JOHNSON, NANETTE ROBERTS, Tufts University
PEDERSON, THORU, Worcester Foundation for Experimental Biology
PEISACH, JACK, Albert Einstein College of Medicine
PERSON, PHILIP, V. A. Hospital, Brooklyn, New York
QUATTROCHI, JAMES J., Ohio State University College of Medicine
REINER, JOHN M., Albany Medical Center
RICE, ROBERT, Carnegie-Mellon University
ROSENBLUTH, RAJA, Simon Fraser University, Canada
ROWLAND, LEWIS P., Neurological Institute, New York
RUESS, LYNNE, Kenyon College
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RUSSELL-HUNTER, W. D., Syracuse University
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SCHWARTZ, MARTIN, University of Maryland, Baltimore County
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SHEMIN, DAVID, Northwestern University
SHEPHARD, FRANK, Deep Sea Research
SHEPRO, DAVID, Boston University
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SINGER, MAXINE, NIH
SLUDER, GREENFIELD, Worcester Foundation for Experimental Biology
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SPECK, WILLIAM, Case Western Reserve University School of Medicine
SPECTOR, A., Columbia University
SPEIGEL, MEL, Dartmouth College
STEPHEN, MICHAEL J., Rutgers University
STEPHENS, RAYMOND, Marine Biological Laboratory
STUNCKARD, HORACE, American Museum of Natural History
SUSSMAN, MAURICE, University of Pittsburgh
TASHIRO, JAY S., Kenyon College
TRAGER, WILLIAM, The Rockefeller University
TWEEDELL, KENYON S., University of Notre Dame
WAINIO, WALTER, Rutgers University
WANGH, LAWRENCE, Brandeis University
WARREN, LEONARD, Instar Institute
WEBB, H. MARGUERITE, Woods Hole, Massachusetts
WEBSTER, LESLIE T., Case Western Reserve Medical School
WEIDNER, EARL, Louisiana State University
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WITTENBERG, BEATRICE A., Albert Einstein College of Medicine
WITTENBERG, JONATHAN B., Albert Einstein College of Medicine
WOLKEN, JEROME J., Carnegie-Mellon University
WORTHINGTON, C. R., Carnegie-Mellon University
ZACKS, SUMNER I., Miriam Hospital
ZELESKI, ILENE, Deep Sea Research
ZIMMERMAN, MORRIS, Merck, Sharp & Dohme Research Laboratories

OTHER RESEARCH PERSONNEL

ALLIEGRO, MARK, SUNY, Buffalo
ANDERSON, CATHLEEN, Syracuse University

AUGUSTINE, GEORGE, University of California, Los Angeles
BAKER, ROBERT G., New York University Medical Center
BEACH, ROBERT L., University of Virginia Medical Center
BETCHAKU, TEIICHI, Yale University School of Medicine
BLUMER, JEFFREY, Case Western Reserve University
BOOKMAN, RICHARD, University of Pennsylvania
BOSS, WENDY, University of North Carolina
BOWER, JAMES, New York University Medical Center
BRADY, SCOTT, Case Western Reserve University
BREITWIESER, GERDA E., University of Texas Medical Branch
BROWN, DOUGLAS, Dartmouth College
CAPUTO, CARLO, National Institutes of Mental Health
CARDEN, M., University of Ottawa, Canada
CARIELLO, LUCIO, Stazione Zoologica, Naples, Italy
CHUNG, MARGARET P., Tufts University Medical School
CLAPIN, D. F., University of Ottawa, Canada
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DIPOLO, RINALDO, Medica Instituto d'Investigacion, Argentina
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FEINMAN, RICHARD D., Downstate Medical Center, Brooklyn
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GIUDITTA, ANTONIO, International Institute of Genetics and Biophysics, Italy
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GOULD, ROBERT, New York Institute of Basic Research for Mental Retardation
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GRIZZLE, RAYMOND, Rutgers University
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HU, SHI LING, Downstate Medical Center, Brooklyn
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KISHIMOTO, TAKEO, National Institute for Basic Biology, Japan
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NADEAU, JOSEPH, The Jackson Laboratories
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ORBACH, HARRY, Yale University School of Medicine
PANT, HARISH, National Institutes on Alcohol Abuse and Alcoholism
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STIMERS, JOSEPH, University of California, Los Angeles
STOCKBRIDGE, NORMAN, New York Medical College
SUGIMORI, M., New York University School of Medicine
SZAMIER, BRUCE, Marine Biological Laboratory
SZENTKIRALYI-SZENT-GYÖRGYI, EVA, Brandeis University
TANGUY, JOELLE, Laboratoire de Neurobiologie
TAYLOR, LAVENTRICE, University of North Carolina
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TIFFERT, T., University of Maryland
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VARNER, JUDITH, Klingelbergstrasse Biocenter, Switzerland
VOSSHALL, LESLIE, Syracuse University
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WANG, LINFANG, Rockefeller Foundation
WEISS, J., Northwestern University School of Medicine
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WHITE, MICHAEL M., University of California, Los Angeles
WHITTEMBURY, JONATHAN, University Peruana, Cayetano
WIENS, T. J., University of Manitoba, Canada
WIRTZ, K. W. A., State University of Utrecht, Netherlands
WU, CHAU H., Northwestern University School of Medicine
YAMAMOTO, DAISUKE, Northwestern University School of Medicine
YOKO, KAREN, Northwestern University School of Medicine
ZAKEVICIUS, JANE, New York University School of Medicine
ZAVILOWITZ, J., Albert Einstein College of Medicine
ZECEVIC, DEJAN, Institute of Biological Research, Belgrade, Yugoslavia

YEAR-ROUND PROGRAMS

(All of Marine Biological Laboratory unless otherwise indicated)

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ATEMA, JELLE
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CROWTHER, ROBERT
D'AVANZO, CHARLENE, Hampshire College
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RIETSMA, CAROL, SUNY, New Paltz
SCHWALBE, KAREN

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TAMM, SIGNHILD
TAYLOR, MARGERY
VALIELA, IVAN
VAN ETTEN, RICHARD
VOIGHT, RAINER
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BRYANT, DONALD
BUCHSBAUM, ROBERT
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CHU, KEVIN
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GOEHRINGER, DALE
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HALL, VALERIE
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HETTENBACH, GAIL
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WILSON, JOHN
WOOD, SUSAN

Undergraduates

ALCESTE, CESAR
CHASE, JAMES
GADZIK, MARY BETH
GLICK, STEPHEN
IANNAZZI, RUTH
KOENIG, PATRICIA
KYRIAZI, CONSTANT
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O'LOUGHLIN, JOHN T.

LABORATORY OF BIOPHYSICS

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Section on Neural Membranes

Staff (of NINCDS-NIH unless otherwise indicated)

ADELMAN, WILLIAM J., JR., *Chief*
CLAY, JOHN R.
DEFELICE, LOUIS J., Emory University
FOHLMEISTER, JURGEN F., University of Minnesota
GOLDMAN, DAVID E., SUNY, Binghamton
HODGE, ALAN J., Marine Biological Laboratory
LEONARD, DOROTHY A.
MUELLER, RUTHANNE, Marine Biological Laboratory
RICE, ROBERT V., Carnegie-Mellon University
STANLEY, ELIS, Johns Hopkins University
TYNDALE, CLYDE L., Marine Biological Laboratory

VOLKMAN, MARY, Marine Biological Laboratory
WALTZ, RICHARD B., Marine Biological Laboratory

Section on Neural Systems

Staff

ALKON, DANIEL L., *Chief*
ACOSTA-URQUIDI, JUAN
COULTER, DOUGLAS, Boston University
FARLEY, JOSEPH, Princeton University
FORMAN, ROBIN
GART, SERGE, University of Vermont
GOH, YASUMASA
HARRIGAN, JUNE, Marine Biological Laboratory
HAY, BRUCE, University of California
JACKLET, JOHN, SUNY, Albany
KUZIRIAN, ALAN M.
KUZIRIAN, JEANNE
LEDERHENDLER, IZJA, Marine Biological Laboratory
LEIGHTON, STEPHEN, NIH
NEARY, JOSEPH T., Marine Biological Laboratory
RICHARDS, WILLIAM, Princeton University
SAKAIBARA, MANABU
SHOUKIMAS, JONATHAN J.
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WOOLF, THOMAS, University of Chicago

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Staff

ADAMS, NANCY L.
ALATALO, PHILIP
BERG, CARL J., JR.
MITTON, JEFFREY B., University of Colorado
ORR, KATHERINE S.

LABORATORY OF CAROL L. REINISCH

Staff

LEAVITT, DALE, Tufts University School of Veterinary Medicine
REINISCH, CAROL L., Tufts University School of Veterinary Medicine
SAKAMOTO, HIDEMI, Tufts University School of Veterinary Medicine

Visiting Investigators

CHARLES, ANN, Tufts University School of Veterinary Medicine
FARLEY, AUSTIN, National Marine Fisheries Service

LABORATORY OF D. EUGENE COPELAND

Staff

BLOCK, BARBARA, Duke University
COPELAND, D. EUGENE

Laboratory of Eric Kandel

Staff

Bidwell, Joseph, Howard Hughes Medical Institute
Capo, Thomas, Howard Hughes Medical Institute
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Kandel, Eric, Columbia University
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Paige, John A., Howard Hughes Medical Institute
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Laboratory of Felix Strumwasser

Staff

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Strumwasser, Felix, California Institute of Technology

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Staff

Crowther, Robert
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Meedel, Thomas H.
Whittaker, J. Richard, Boston University/Marine Biological Laboratory

Visiting Investigators

Miyamoto, David, Seton Hall University

Laboratory of Judith P. Grassle

Staff

Gelfman, Cecilia
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Mills, Susan

Laboratory of Marine Animal Health

Staff

Abt, Donald, University of Pennsylvania
Leibovitz, Louis, Cornell University, *Director*
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Staff

De Terra, Noel
Moss, Ann

Laboratory of Osamu Shimomura

Staff

Nemeth, Edward
Shimomura, Akemi
Shimomura, Osamu, Boston University School of Medicine

Visiting Investigators

Anctil, Michel, University of Montreal, Canada
La, Sung, William Paterson College of New Jersey

Laboratory of Raymond E. Stephens

Staff

Shoukimas, Jonathan J.
Stephens, Raymond E., Marine Biological Laboratory/Boston University School of Medicine
Stommel, Elijah, Marine Biological Laboratory/Boston University School of Medicine

Laboratory of Sensory Physiology

Staff

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Cook, Patricia B.
Cornwall, Carter, Boston University School of Medicine
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Fein, Alan
Harosi, Ferenc I.
Hashimoto, Yoko, Tokyo Women's Medical College, Japan
Levine, Joseph S.
Lipetz, Leo, Ohio State University
MacNichol, Edward F., Jr., *Director*
Mansfield, Richard, Boston University School of Medicine
Payne, Richard
Szuts, Ete Zoltan

Laboratory of Shinya Inoué

Staff

Akins, Robert E., University of Pennsylvania
Brown, Carolyn R., University of Pennsylvania
Inoué, Shinya, Marine Biological Laboratory/University of Pennsylvania
Inoué, Theodore D., Cornell University
Lutz, Douglas A., University of Pennsylvania
Woodward, Bertha M.

Visiting Investigators

Ellis, Gordon W., University of Pennsylvania
Horn, Edward, University of Pennsylvania
Okazaki, Kayo, Tokyo, Japan
Otter, Timothy, Albert Einstein School of Medicine
Schweitzer, Peter, University of Pennsylvania
Tilney, Lewis G., University of Pennsylvania
Woodruff, Richard I., West Chester State College

Laboratory of Thomas S. Reese

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Reese, Thomas S.
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National Foundation for Cancer Research

Staff

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Szent-Györgyi, Albert, *Director*

National Vibrating Probe Facility

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Jaffe, Lionel, Purdue University, *Director*
Scheffey, Carl E.
Shipley, Alan M.

Visiting Investigators

Goodall, Harry, University of Cambridge, U. K.
Robinson, Kenneth, University of Connecticut
Saltzman, Charles, University of North Carolina

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Sechoka, Elizabeth
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Steudler, Paul A.
Stone, Thomas A.
Turner, Andrea
Woodwell, George M., *Director*

Trainees

CAVANAUGH, COLLEEN, Harvard University
SAMPOU, PETER, University of Rhode Island

XI. HONORS

FRIDAY EVENING LECTURES

PITTENDRIGH, COLIN, Hopkins Marine Station, Stanford University, 24 June, *"The Structure and Evolution of Circadian Programs"*
KRAVITZ, EDWARD A., Harvard University School of Medicine, 1 July, Lang Lecture, *"The Well-Modulated Lobster: Neurohormones and Aspects of Lobster Behavior"*
GURDON, JOHN B., M. R. C. Laboratory of Molecular Biology, 8 July, *"Clones of Frogs and Some Principles of Development"*
LAND, EDWIN H., Rowland Institute for Science, 15 July, *"Recent Advances in Retinex Theory and Some Implications for Cortical Computations"*
PURVES, DALE, Washington University School of Medicine, 21, 22 July, Forbes Lectures, I. *"Formation and Maintenance of Synaptic Connections Between Neurons: Quantitative Aspects;"* II. *"Formation and Maintenance of Synaptic Connections Between Neurons: Qualitative Aspects"*
BERG, PAUL, Stanford University School of Medicine, 29 July, *"The Prospects of Gene Replacement Therapy in Human Disease"*
KAMINER, BENJAMIN, Boston University School of Medicine, 5 August, "Albert Szent-Györgyi: *Search and Discovery*"
JEFFERY, WILLIAM R., University of Texas, 19 August, E. E. Just Lecture, *"Control of Egg Polarity: New Dimensions to an Old Problem"*
JANNASCH, HOLGER, Woods Hole Oceanographic Institution, 26 August, *"Plant Life in the Deep Sea"*

ASSOCIATES' LECTURE

WILSON, E. O., Harvard University, 12 August, *"The Social Life of Ants"*

SPECIAL LECTURES

SMITH, FEDERICK E., Harvard University, 13 July, Charles A. Lindbergh Lecture in Ecology, *"Niche Theory, Genetic Systems, and the Survival of Species"*
BETHE, HANS A., The Floyd R. Newman Laboratory of Nuclear Science, Cornell University, 31 August, *"The Arms Race"*

STEPS TOWARD INDEPENDENCE FELLOWS

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SCHNEIDER, E. GAYLE, University of Nebraska Medical Center
SILVER, ROBERT, University of Chicago Medical School
WOLNIAK, STEPHEN, University of Maryland

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BERGGREN, RUTH, Oberlin College

COBLER, SUE, Northeastern University
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GAIDE, MICHAEL, Free University of Berlin, West Germany
KESSING, BAILEY, New College
LASTA, MARIO, Institute of Marine Biology, Argentina
LIEBMAN, MATTHEW, SUNY, Stony Brook
PASCUAL, MARCELA, Institute of Marine Biology, Argentina
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Reference: *Biol Bull.* **167**: 94–119. (August, 1984)

DEVELOPMENT OF ASYMMETRY IN THE NEUROMUSCULAR SYSTEM OF LOBSTER CLAWS

C. K. GOVIND

Department of Zoology, Scarborough Campus, University of Toronto, West Hill, Ontario, Canada, M1C 1A4

ABSTRACT

The paired claws of the lobster *Homarus americanus* which are symmetrical in form and function in the larval and early juvenile stages gradually transform into a slender, fast-acting cutter claw and a stout, slow-acting crusher claw during later juvenile and adult stages. Correspondingly changes occur in the neuromuscular system of the claws. The paired claw-closer muscles are initially symmetrical in their fiber composition and consist of a band of fast fibers sandwiched on either side by slow fibers. During development one of the muscles transforms into a cutter with a majority of fast fibers and a small ventral band of slow fibers and the other muscle transforms into a crusher with only slow fibers. The firing pattern of the juvenile fast closer excitor motoneuron consisting of high frequency, long duration bursts, is essentially retained in the adult crusher but changed in the adult cutter to low frequency, short duration bursts. In the paired juvenile closer muscles almost all fibers receive mixed innervation from both fast and slow axons whereas in the adult cutter muscle innervation by the fast axon predominates while in the crusher both are equitably distributed. The development of asymmetry in the closer muscle is regulated by impulse-mediated muscle tension though how the neural asymmetry arises is unknown, but amenable to experimentation.

INTRODUCTION

The body plan of many higher animals from annelids to vertebrates is characterized by symmetry of the left and right sides. Within this bilaterally symmetrical organization, however, asymmetries arise manifested most dramatically by cerebral lateralization in humans (Corballis and Morgan, 1978), vocalization in songbirds (Nottebohm, 1977), and cheliped laterality in crustaceans (Przibram, 1901). Despite the tremendous interest throughout the ages in human laterality we still do not understand how it or any of the other biological asymmetries in the animal world is acquired. One of the more compelling hypotheses put forward by Corballis and Morgan (1978) attributes cerebral lateralization to a left-right maturational gradient. According to this scheme both sides are equipotent initially, but mature at different rates subsequently, with the left leading and at the same time suppressing the right; thereby resulting in the left cerebral hemisphere being dominant for speech and verbal processes while the right deals with non-verbal input. This also explains why when the left side is damaged or lesioned the right side is more disposed to take over its function than vice-versa. Among certain songbirds sectioning of the left hypoglossal nerve, but not the right, severely disrupts the singing pattern and demonstrates the lateralization of singing which includes not only the efferent pathway but the associated nuclei in the brain (Nottebohm, 1977). However, the right side can take over control of singing if the

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left hypoglossus is sectioned before the onset of spring song suggesting that both sides have the capacity for singing but its expression is normally limited to the left side. These examples of asymmetry point to a bias during development which may be profitably studied among crustaceans such as the lobster *Homarus americanus*.

The paired chelipeds or claws of the adult lobster are asymmetric in form and function consisting of a greatly enlarged, slow acting, and powerful crusher claw either on the left or right side, and a more slender, fast-acting cutter claw on the opposite side (Herrick, 1895). Yet in the larval and early juvenile stages the paired claws are symmetrical and equipotent. The neuromuscular system within these claws has received considerable attention because of their relative simplicity: there are only two muscles each innervated by few motoneurons (Wiersma, 1955). It is the development of asymmetry in the neuromuscular system of the paired claws that is reviewed here as part of an ongoing study to understand the biological basis of asymmetry.

Development of Asymmetric Claws

The natural history of the east coast lobster *Homarus americanus* is given in narrative detail in the two voluminous works of Herrick (1895, 1911). The adult female bears eggs every second year. Following a molt usually in July she copulates with a male and subsequently extrudes fertilized eggs. These eggs are carried attached to the swimmerets until the following spring when they hatch as myesid larvae. All three larval stages are planktonic, swimming by means of fan-shaped expodites on their thoracic appendages (Neal *et al.*, 1976). In all three larval stages the paired claws are symmetrical in form (Fig. 1) and slightly larger than the walking legs. They have a few conspicuous sensory bristles but do not have any teeth on their biting surfaces which is characteristic of the adults. At the molt to the 4th stage, which is the first juvenile stage, the animal transforms to a diminutive lobster in that it loses the expodites, and now swims by means of its swimmerets located on the abdomen. At

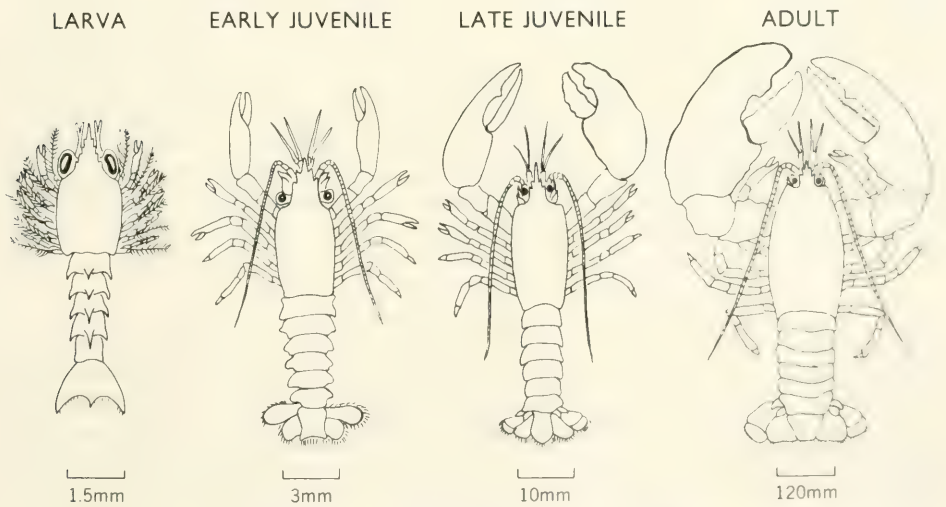


FIGURE 1. Development of the paired claws of the lobster beginning from a symmetrical condition in the larval (1st) and an early juvenile (4th) stage to an increasingly asymmetrical condition in a late juvenile (12th) and an adult stage. Magnification: larva 13×; early juvenile 7×; late juvenile 2×; adult 0.2×.

the same time the claws elongate disproportionately compared to the walking legs and are held extended in front of the animal. The biting surfaces in particular are covered with sensory hair and show the first signs of dentition, usually a single central tooth on the pollex. The paired claws in the 4th, 5th, and 6th stage are symmetrical in form but begin to differentiate in the succeeding stages with the putative cutter claw remaining long and slender and the putative crusher becoming short and stout. The other characteristic change concerns the central tooth on the pollex which remains sharp and narrow (incisor-like) in the cutter while becoming rounded and broad (molar-like) in the crusher. The differentiation in external morphology continues until in the adult the paired claws consist of a distinct cutter and crusher claw. Indeed the claws appear to continue elaborating their distinct external form as the asymmetry becomes even more striking in large adults. It is known that the claws grow in a positive allometric fashion compared to the rest of the body (Lang *et al.*, 1977c) throughout the life of the lobster.

Less is known about the development of functional differentiation between the paired claws. Casual observation in the larval stage show the claws to be used in grasping food. This is supplemented in the early juvenile stages by "meral display" in which the paired claws are held extended and open in a threatening or defensive posture. In these early stages the claw closes at a variety of speeds ranging from approximately 50 to 400 ms (Hill and Govind, 1984). Both claws display this range of closing speeds. It is only in late juveniles and early adults that a clear distinction in closing speeds occurs between the asymmetric claws (Govind and Lang, 1974, 1979). Now the cutter claw displays both fast and slow closing speeds while the crusher closes only slowly. In isolated claws stimulation of the fast closer excitor (FCE) axon with 2 impulses 6.5 ms apart closes the cutter claw in 20 ms while in the crusher claw the homologous axon required 8 impulses, 5 ms apart to cause closing in 90 ms. The closing behavior fatigues more readily and at a lower frequency of stimulation of the FCE in the cutter than in the crusher claw. An essentially similar differentiation in closing behavior was seen between the crusher and cutter claws with stimulation of the slow excitor (SCE) axon. Thus tonic contractions were observed at a lower stimulus frequency and they fatigued more rapidly in the cutter than in the crusher claw. Overall closing of the crusher claw is much slower and more powerful than in its cutter counterpart with stimulation of the homologous motoneurons. The difference in closing behaviors between the paired asymmetric claws is seen in all sizes of adult lobsters including some very large animals, thus suggesting that the functional differentiation is maintained throughout the life span of the lobster. How this functional dissimilarity develops will be traced by examining the muscular and neural substrates governing claw behavior.

Development of Muscle Asymmetry

The lobster claw represents a relatively simple motor system having only two antagonistic muscles (Fig. 2). Both muscles are bipinnate in form and run the length of the propus. The opener muscle is relatively small occupying 10% of the claw muscle mass while the massive closer makes up the other 90%. The opener muscle is situated distally and its contraction opens the dactyl: the closer muscle closes the dactyl on the pollex. Most of the work on the development of asymmetry has been done on the closer muscle because it is responsible for the striking differences in closing behavior of the cutter and crusher claws. On the other hand such differences are not obvious in the opening behavior and consequently the opener muscle has received scant attention.

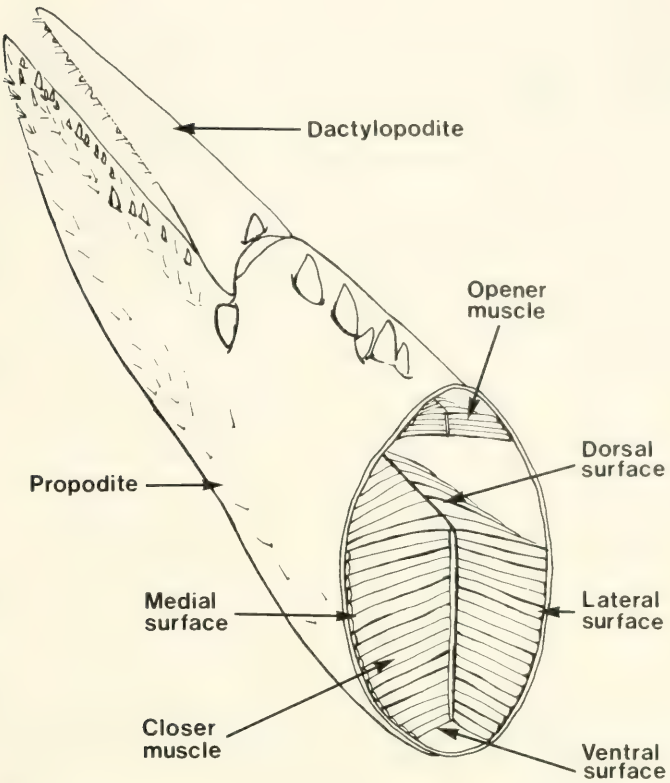


FIGURE 2. Cut-away diagram of an adult cutter claw showing the location and relative size of the antagonistic opener and closer muscles (from Govind and Lang, 1974).

Closer muscle

The development of the closer muscle in the paired claws has been extensively investigated especially with regard to its fiber composition using contractile, structural, histochemical, and biochemical characteristics. The overall picture obtained from all these studies is the symmetry in fiber composition of the paired muscles in the larval and early juvenile stages with the gradual differentiation into a cutter muscle with predominantly fast fibers and some slow fibers and a crusher muscle with all slow fibers.

Structural properties. Unlike vertebrate muscle in which the different fiber types of fast-twitch and slow-twitch have a uniform sarcomere length (SL) of 2–4 μm, crustacean muscle has a wide range of SL from 2–20 μm (Govind and Atwood, 1982). Early studies by Atwood and his collaborators (reviewed by Atwood, 1967, 1973) established that short SL (2–4 μm) fibers are fast-contracting while long SL (>6 μm) fibers are slow-contracting. Using this scheme the fiber composition of the paired closer muscle was determined during development (Jahromi and Atwood, 1971; Goudey and Lang, 1974; Lang *et al.*, 1977a, b, c; Govind and Lang, 1978; Costello and Lang, 1979) and is summarized in Table I and Figure 3. The grouping of sarcomeres into the three categories of short <4 μm, intermediate 4–6 μm, and long >6 μm, in Table I was based on the prevailing dogma that these represented respectively fast,

TABLE I

Fiber composition based on sarcomere length of the paired claw closer muscles during development of the lobster

Stage	No. of animals	Length of animal (mm)	% of fiber types based on sarcomere length (μm)*					
			Claw I (cutter)			Claw II (crusher)		
			Fast <4	Intermediate 4–6	Slow >6	Fast <4	Intermediate 4–6	Slow >6
Larval								
1	3	7.5	39	58	3	29	68	3
2	3	8.5	43	35	2	40	56	4
3	5	10	54	45	21	25	54	21
Early Juvenile								
4	7	12	36	5	59	26	3	71
5	5	14	50	1	49	27	1	72
Late Juvenile								
6	4	16	56	0	44	21	1	78
11	2	32	73	1	26	23	1	76
13	1	39	64	0	36	0	0	100
15	1	55	82	0	18	4	0	96
Adult								
?	1	250	63	0	37	0	4	96

* The number of fibers sampled from each closer muscle was 30 for the larval stages, 60 for the juvenile 4th stage, and 90 for the remainder (from Lang *et al.*, 1977a, b; and Govind and Lang, 1978).

intermediate, and slow fiber types (Atwood, 1967, 1973). According to the scheme the first two larval stages have predominantly short and intermediate SL fibers. There is a substantial increase in the number of long SL fibers at the 3rd (larval) stage, and again at the 4th and 5th (early juvenile) stages. These increases occur at the expense of the intermediate SL fibers so that by the 5th stage there are few fibers of intermediate SL. These data show a lengthening of the SL from intermediate to long during development of the larval and early juvenile stages. Such lengthening of the sarcomeres appears to be a normal process of crustacean muscle development (Bittner, 1968; Govind *et al.*, 1974; Bittner and Traut, 1978). Over and above this growth-related process, the closer muscle shows two distinct populations of relatively short and long SL fibers in the larval and early juvenile stages (Table I). This dichotomy is graphically represented in the histograms of SL (Fig. 3) where for the larval stage, the fibers separate into the categories of <4 μm and >6 μm. Development of the closer muscle up to the early (4th and 5th) juvenile stages shows a distinct separation of short and long SL fibers. This distribution is seen in both of the paired muscles.

The asymmetry between the paired muscles occurs in the succeeding juvenile stages. Beginning with the 6th stage the population of short SL (<4 μm) increases in one of the paired claws while the population of long SL (>6 μm) fibers increases in the other claw. As a result of these changes in SL, the cutter muscle ends up with predominantly (60–80%) short SL fibers and the remainder long SL fibers while the crusher muscle ends up with all long SL fibers. The asymmetry of the paired closer muscles characteristic of the adult is usually established by the 13th stage which represents the first year of juvenile development. It takes between 5–7 years for lobsters to mature into adults (Hughes *et al.*, 1972).

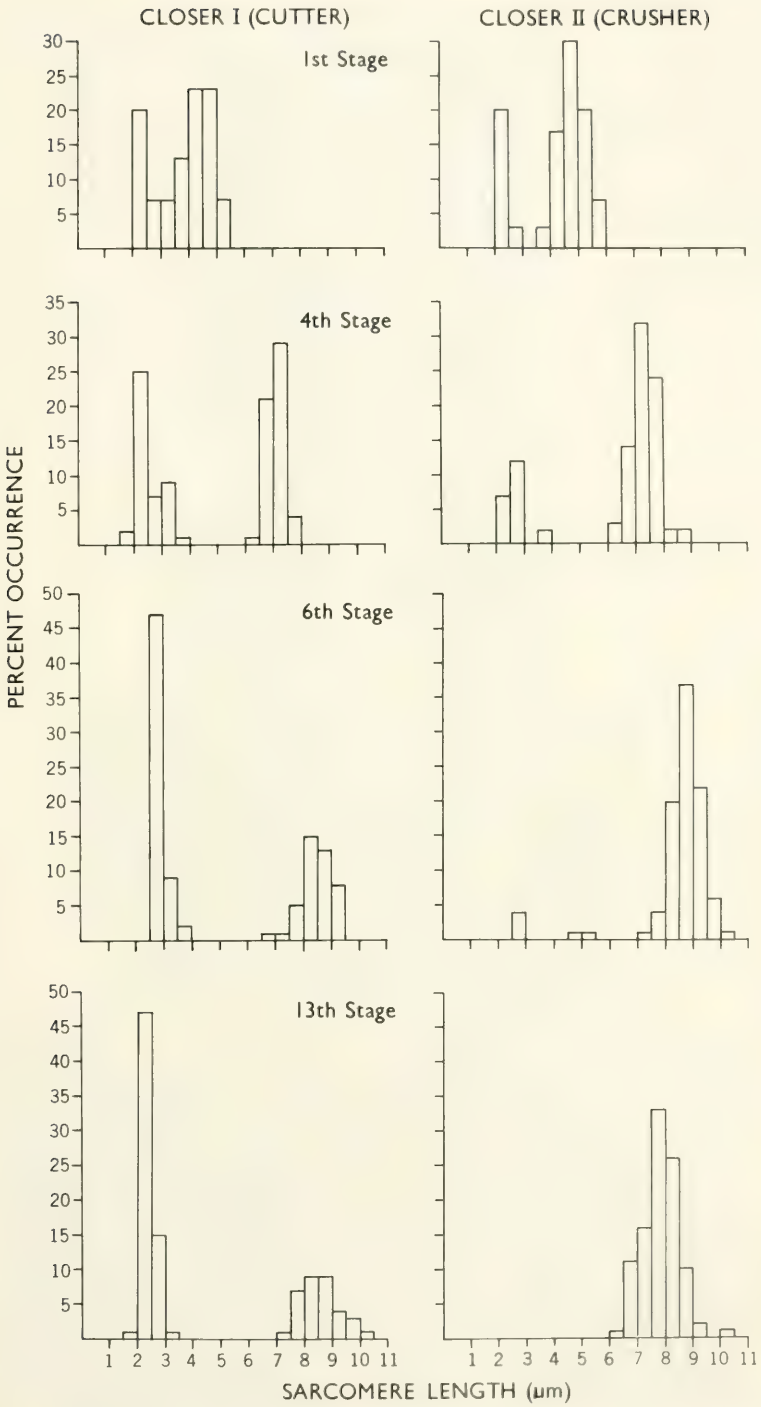
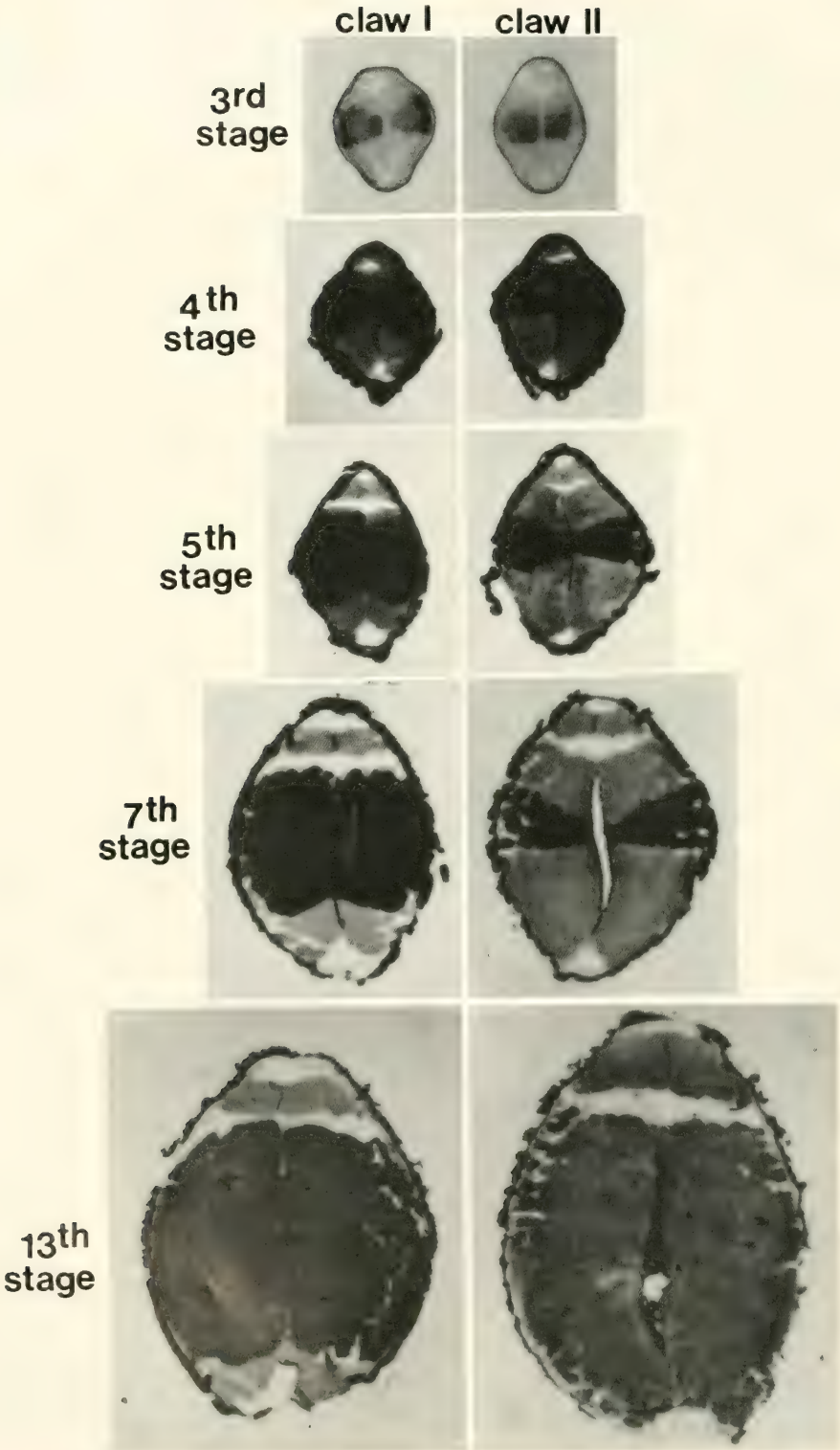


FIGURE 3. Histograms of percent occurrence of muscle fiber types based on sarcomere length in the paired claw closer muscles during development as represented by a larval (1st) and several juvenile (4th, 6th, 13th) stages. Number of fibers sampled for each claw is 30 for the 1st stage, 60 for the 4th stage, and 90 for the remaining stages (from Lang *et al.*, 1977a, b, Govind and Lang, 1978).


claw I
claw II
3rd stage
4th stage
5th stage
7th stage
13th stage

The transformation of the paired claw closer muscles from the symmetrical to the asymmetrical condition involves the acquisition of short SL, presumably fast, fibers in the putative cutter claw and of long SL, presumably slow, fibers in the putative crusher claw. Since no evidence for degenerating fibers has been found, the changeover to short and long SL fibers in the appropriate claws must be due to the transformation of existing fibers. The transformation from short to long SL fibers in the development of the crusher claw may be explained by the lengthening of sarcomeres: a process which has been amply demonstrated among crustacean muscle fibers. However, the transformation of long to short SL fibers during development of the cutter claw is not as easily explained. They could arise by longitudinal splitting of existing short SL fibers; a mechanism which has been suggested to account for growth of a lobster leg muscle (El-Haj *et al.*, 1984), or the short SL fibers could arise by transverse splitting of sarcomeres at their H-bands as has been shown to occur in an adult crab muscle (Jahromi and Charlton, 1978).

Histochemical properties. Among vertebrates determination of muscle fiber types using enzyme histochemistry for the detection of myofibrillar adenosinetriphosphatase (ATPase) activity is well established (review by Burke, 1981). Such histochemical techniques have only more recently been applied to crustacean muscle (Ogonowski and Lang, 1979) where fast muscle stains more intensely than slow since the specific activity of myofibrillar ATPase of crustacean fast muscle is two to three times greater than that of slow muscle (Hajek *et al.*, 1973; Lehman and Szent-Györgyi, 1975).

The differentiation of fiber types in the paired closer muscles was followed in a larval and several juvenile stages (Fig. 4) (Ogonowski *et al.*, 1980). In the 3rd larval stage the paired muscles were symmetrical in their fiber composition consisting of a central band of dark-staining fast fibers sandwiched by light-staining, slow fibers on the dorsal and ventral surfaces. Histochemistry of the 1st and 2nd stage larval claws revealed little if any staining for ATPase in the muscles suggesting that the fibers had little (if any) of this enzyme in these early developmental stages. The symmetry in fiber composition between the paired muscles is also seen in the juvenile 4th stage, though occasionally slight asymmetries in the width of the central dark-staining band are present (Fig. 4). In the 5th stage one of the claws has the central fast band consistently broader than that of its counterpart claw. This is the putative cutter claw where the fast fibers continue to be elaborated over most of the closer muscle except for a narrow ventral strip in the succeeding juvenile stages until the process is completed by the 9th–10th stage. The other claw differentiates into the crusher by the expansion of slow fibers dorsally and ventrally and the diminution of the central fast band until about the 13th stage when the muscle is composed of all slow fibers. Thus at the end of the first year of development the paired closer muscles are differentiated into their asymmetric condition. The cutter muscle has predominantly fast fibers and a small ventro-lateral band of slow while the crusher muscle has all slow fibers. Among the slow fibers in both claws there is a small sub-population located distally which are slower than the remaining majority (Kent and Govind, 1981.)

The development of asymmetry in the paired closer muscles from a symmetric condition occurs by the transformation of slow to fast fibers in the putative cutter claw and of fast to slow in the putative crusher claw. This is suggested by the observation of fibers with an intermediate staining intensity than that characteristic of fast and

FIGURE 4. Representative cross-sections stained for myofibrillar ATPase activity showing distribution of fast (dark-staining) and slow (light-staining) fibers during development of the paired closer muscles in a larval (3rd) and several juvenile (4th, 5th, 7th, 13th) stage lobsters. The small dorsally situated opener muscle retains its slow (light-staining) character throughout development (from Ogonowski *et al.*, 1980).

slow fibers. Thus in the putative cutter muscle these intermediate type fibers were found in the dorsal region which is destined to become fast while in the putative crusher muscle they were found in the central region which is destined to become slow. (Fig. 4). Such changeovers in the enzymatic profile of fibers have been shown to occur between the fast-twitch and slow-twitch fibers in vertebrate muscle and to be under the direction of the innervating motoneurons (reviewed by Guth, 1968; Gutmann, 1976; Harris, 1974; Jolesz and Sreter, 1981).

Biochemical properties. The protein composition of the closer muscle in juvenile and adult lobsters has been examined using gel electrophoresis (Costello and Govind, 1984). Adult fast and slow muscle have several proteins in common and these are listed along with their molecular weights as follows: (Fig. 5) myosin heavy chain (HC, 154K), two myosin light chains (LC1, 20K in doublet form and LC2, 16K), actin (A, 41K), tropomyosin (TM, 34K), and a protein tentatively identified as α-actinin at 92K. Another major protein tentatively identified as paramyosin (P) differs in molecular weight between fast and slow muscle at 99K and 96K respectively. Apart from these common proteins, adult fast and slow muscle have proteins unique to

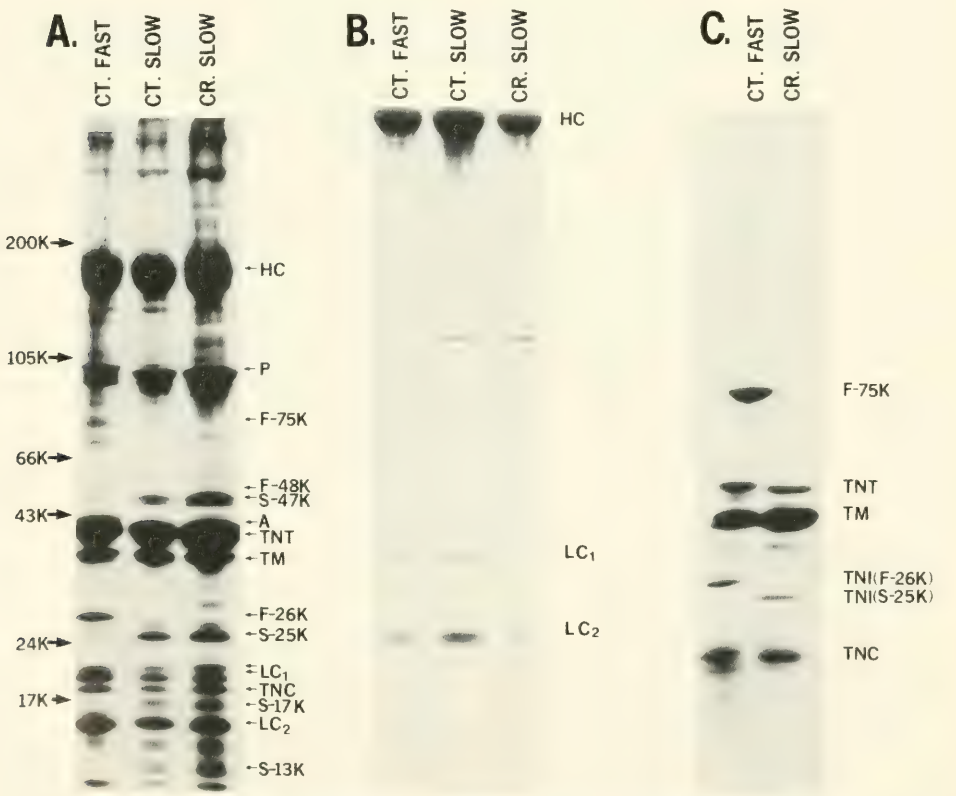


FIGURE 5. Electrophoretic protein patterns of fast and slow muscle from the cutter (CT) and crusher (CR) claw closer muscle of an adult lobster. A, Whole myofibrillar homogenate showing the common proteins such as myosin heavy chain (HC), two myosin light chains (LC1, LC2), actin (A), paramyosin (P), tropomyosin (TM), troponin-C (TNC), and troponin-T (TNT). Proteins unique to fast (F) and slow (S) muscle are so indicated at their respective molecular weights. B, Myosin extract showing heavy and light chains. C, Troponin-tropomyosin extract showing several unique proteins and troponin-I (TNI) (from Costello and Govind, 1984).

themselves. There were three such bands in the electrophoretic pattern for fast muscle seen at F-75K, F-48K and F-26K in Figure 5 and four unique bands for slow muscle at S-47K (doublet form), S-25K, S17K, and S-13K. One of these unique proteins in each fiber type, F-26K in fast muscle and S-25K in slow muscle corresponds to the regulatory protein troponin-I (Fig. 5) Other regulatory proteins include troponin-T (TNT) normally masked by actin and troponin-C (TNC) and tropomyosin (TM) which are common to both fast and slow muscle (Fig. 5).

The earliest stage during development of the paired closer muscles examined electrophoretically was the 4th juvenile stage when the muscles are symmetric in fiber composition. At this stage the closer has almost all of the major proteins common to both adult fast and slow muscle viz. myosin heavy and light chains, actin, paramyosin, and tropomyosin (Fig. 6). A high molecular weight protein at 290K which is common to both types of adult muscle is lacking in the 4th stage muscle. More significantly, however, is the lack of all proteins unique to fast muscle (F-75K, F-48K, and F-26K) and of one protein unique to slow muscle (S-13K) in this juvenile muscle. Furthermore the slow muscle protein S-47K is present in a singlet form in the 4th stage muscle and not in the doublet form characteristic of the adult muscle. These missing proteins are present in the 10th stage muscle, except for S-13K which is still absent in the cutter slow muscle.

The major proteins common to both fast and slow muscle are present in the first juvenile form (4th stage) as are also three of the four proteins unique to slow muscle.

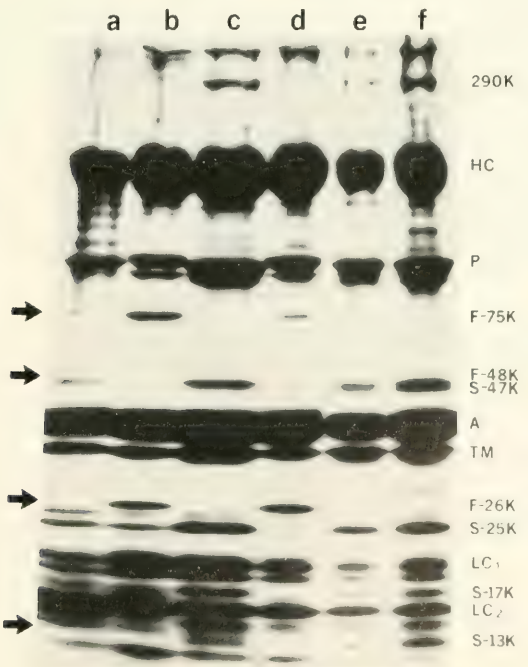


FIGURE 6. Differentiation of the electrophoretic protein pattern of the claw closer muscles. Lane a: undifferentiated muscle of juvenile 4th stage. Lanes b, c: differentiated cutter (fast and slow fibers) and crusher (slow fibers) muscles respectively of juvenile 10th stage. Lanes d, e, f: fully differentiated cutter fast, cutter slow, and crusher slow muscles respectively of an adult. Abbreviations as in Figure 5 (from Costello and Govind, 1984).

During juvenile development the missing unique proteins of fast and slow muscle are expressed. Since some of these unique fast proteins (F-26K, and F-75K) are tentatively identified as troponin I and troponin-tropomyosin complex respectively, their belated appearance suggests a gradual maturation of the regulatory mechanism governing contraction of fast muscle. Moreover, the appearance of these unique proteins during juvenile development not only signals the activation of new genes but underscores the fact that the muscle fibers are differentiating into their adult character.

These biochemical studies do not address the question of how and when the paired closer muscles become asymmetric. In order to answer these questions, individual fibers or at most a small group of fibers taken from areas of the closer muscle known to be either fast or slow according to structural and histochemical tests would have to be analyzed for their protein composition in the first year of development *i.e.,* from the 4th to the 13th stage. This will reveal the protein composition of fibers which are transforming from fast to slow and vice versa.

Contractile properties. The contractile behavior of individual fibers in the adult cutter and crusher muscles has revealed a wide spectrum which has been conveniently grouped into fast, slow, and intermediate types (Jahromi and Atwood, 1971; Costello and Govind, 1983a). Thus fast-follower fibers have a rapid rise to peak tension which is maintained at a plateau and a rapid decay (Fig. 7). Slow-follower fibers show a gradual and continual increase in tension with a decay phase that is equally slow. The intermediate fibers showed a mixture of the tension properties of fast and slow fibers by having an initial rapid rise time followed by a slower rise time. The wide range of contractile behavior encompassed by these three arbitrary categories is seen in the rise time of fibers which extends between 50 to 800 ms for both adult muscles. In larval and early juvenile lobsters the rise time of fibers was between 50 to 400 ms. The slower rise times characteristic of the adult fibers is not present in the 4th juvenile stage and must be acquired during subsequent juvenile development. Indeed there are few slow-follower type fibers in the 2nd, 3rd, and 4th stage muscle, the majority being intermediate and fast-follower types. In the differentiation to asymmetric muscles the slow fiber population increases at the expense of the intermediate fibers in the cutter claw and of the fast fibers in the crusher claw judging by the distribution of these three fiber types during development (Table I).

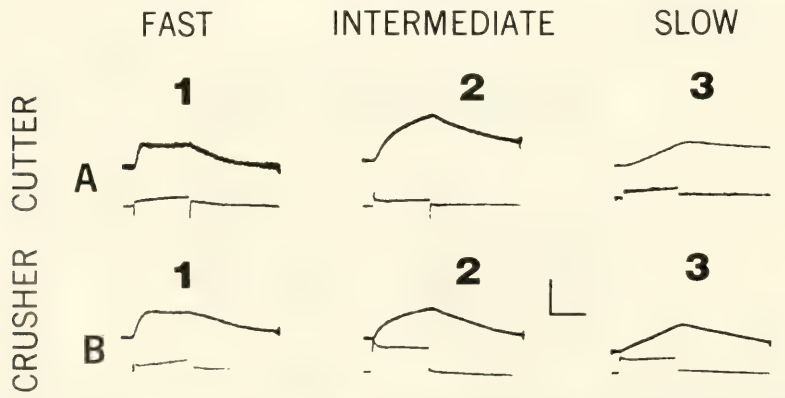


FIGURE 7. Contractile responses of single muscle fibers (upper trace) to short, 800 ms, depolarizing pulses (lower trace) in cutter and crusher claw closer muscles showing representative fast, intermediate, and slow types. Calibration: vertical 5 mg; horizontal, 400 ms. (from Costello and Govind, 1983a).

As a result, the adult crusher muscle has more intermediate and slow fibers and less fast fibers than its cutter counterpart. This would account for the fact that in the intact animal both claws display a wide range of movements from brief, rapid twitches to prolonged, slow contractions (Costello *et al.*, 1984). The asymmetry in contractile types between the paired muscles is therefore in the relative proportion of the three types and not in the fiber types themselves. Given this fact it is interesting to correlate the contractile behavior of these fibers with their SL, ATPase activity and innervation in order to obtain a more comprehensive picture of muscle asymmetry.

Such a correlation (Table II) made for groups of fibers, shows broad agreement with the idea that fast-contracting fibers have high ATPase levels, low oxidative capacities, and short SL, while slow-contracting ones have low ATPase levels, high oxidative capacities and long SL. On an individual basis this three-way correlation does not necessarily hold; *e.g.*, in the crusher all fibers have long SL, low ATPase levels yet can contract rapidly. Finally, when the motor innervation of these bundles of fibers is considered with their other properties, the bundles are seen to be functionally specialized, some for fast, brief contractions (such as the cutter dorsal and proximal bundles) and others for slower, more sustained contractions (such as the central distal bundles).

Opener muscle

As an antagonist to the closer muscle, the opener muscle elevates the dactyl in preparation for the closing action. As such it performs a necessary function, considerably limited in scope, which is reflected by the small size of the muscle compared to the closer. Not surprisingly it has received little attention in the adult claws and none whatsoever during development of the claws.

Structural properties. The frequency histogram of SL from the adult muscles (Fig. 8) shows a range between 6–9 μm for the cutter and 9–11 μm for the crusher, with no overlap between them (Govind *et al.*, 1981). Though the SL of fibers in both adult muscles is >6 μm, the mean SL of the cutter muscle at 8 μm is significantly shorter than that of its counterpart muscle at 10 μm (Fig. 8). Clearly the paired muscles are

TABLE II

Correlation of contractile (rise time) histochemical (ATPase and oxidative capacity) and structural (sarcomere length) properties of closer muscle in different regions of the paired cutter and crusher claws (from Costello and Govind, 1983a)

	Rise time (ms)	ATPase activity	Oxidative capacity	Sarcomere length
Cutter muscle				
dorsal	95	high	low	short
proximal	80	mixed	mixed	short
ventral	236	low	high	long
proximal ventral	326	low	high	long
central distal	489	very low	very high	long
Crusher muscle				
dorsal	232	low	high	long
proximal	189	low	high	long
ventral	256	low	high	long
proximal ventral	379	low	high	long
central distal	554	very low	very high	long

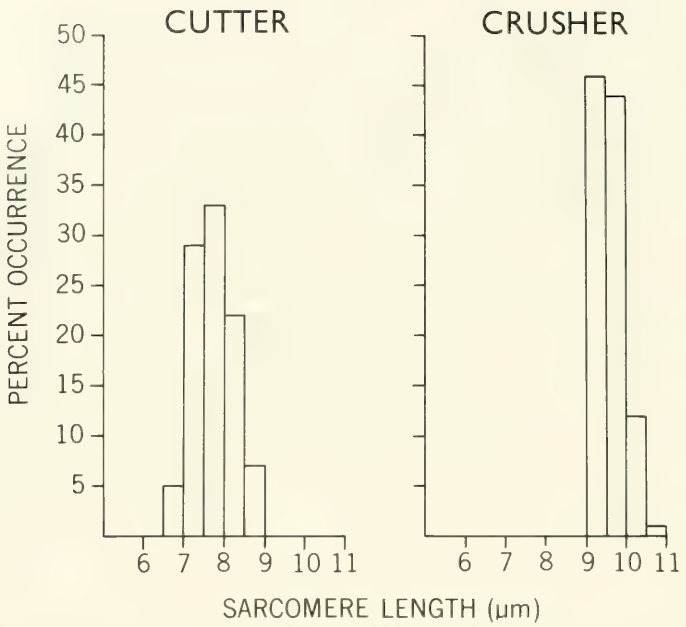


FIGURE 8. Histogram of percent occurrence of muscle fiber types based on sarcomere length in the paired claw opener muscles of an adult lobster. Number of fibers sampled is 158 for each claw (from Govind *et al.*, 1981).

asymmetric in SL though the asymmetry is much more subtle than that seen for the closer muscle.

Histochemical properties. Since cross-sections of the entire claw were taken for histochemistry of the closer muscle (Fig. 3), the opener muscle was always included. The opener muscle showed low specific activity of myofibrillar ATPase typical of slow muscle judging by the light staining compared to the fast fibers of the cutter closer muscle. The staining pattern remains virtually unchanged between the paired claws during development resulting in the symmetry seen in the adult cutter and crusher muscles. Proximal slow fibers of both opener and closer muscles in both claws stain less intensely for ATPase than the remainder (Kent and Govind, 1981), which suggests subdivision within the slow category.

Biochemical properties. The electrophoretic protein pattern of the opener muscle is similar in both adult cutter and crusher claws and resembles the pattern of the slow fibers from the closer muscle (cf. Fig. 5). Thus all the unique proteins of the slow fibers of the closer muscle are found in the opener muscle as well as those represented by the bands at S-47K, S-25K, S-17K, and S-13K. The only difference is the presence of an unidentified protein at 122K which is not found in the closer muscle.

DEVELOPMENT OF NEURONAL ASYMMETRY

The innervation of the limb muscles in crustaceans is well established from the classical work of Wiersma (1961). The claw closer muscle is supplied by three motor axons (two excitors and an inhibitor) which are well-characterized in the adult and whose development has been followed. The claw opener muscle receives only two

axons (an excitor and an inhibitor) which have not been as well studied as the closer axons. In addition there are the large numbers of different sensory receptors landscaping the claw for which little information is available.

Motoneurons to closer muscle

Number and type. The two excitor axons to the adult muscle are differentiated into a fast closer excitor (FCE) and a slow closer excitor (SCE) on the basis of the contractions they evoke (Wiersma, 1955). Though these contractions are qualitatively similar between the paired claws, those to the cutter are more rapid and fatigue more readily than those to the crusher (Govind and Lang, 1974, 1979). Thus the homologous motoneurons are asymmetric in the adult.

Excitatory innervation of the closer muscle is present at the time of hatching with well-defined neuromuscular terminals containing synaptic vesicles and presynaptic dense bars (Fig. 9) denoting active sites of transmitter release at synapses (King and Govind, 1980). The number and type of excitatory axons is however not known in these 1st larval stages. Two excitor axons are physiologically identifiable in the 2nd larval stage with one of them being reminiscent of the FCE (Hill and Govind, 1984). By the 3rd larval stage, the two axons are sufficiently well-differentiated to be recognizable as putative FCE and SCE axons and their physiological identity is firmly established in the succeeding juvenile stages (Costello *et al.*, 1981), though exactly when the homologous motoneurons diverge into cutter and crusher types is not known. Morphologically the motoneurons mature within the first year of development so that by the 10th juvenile stage they resemble their adult counterparts (Fig. 10) (Hill and Govind, 1983). The general form for both FCE and SCE neurons is similar, consisting of an antero-ventrally located soma from which a single neurite rises vertically to the dorsal surface of the ganglion. The neurite courses diagonally across the ganglion to the second root which it enters as an axon. Dendritic branches which

FIGURE 9. Excitatory neuromuscular terminal (E) recognized by spherical synaptic vesicles (v) adjacent to an inhibitory terminal (I) which contains irregularly-shaped vesicles in the juvenile 4th stage claw closer muscle. These two types of terminals also occur in the larval 1st stage and adult muscle. Magnification 20,000×. (from King and Govind, 1980).

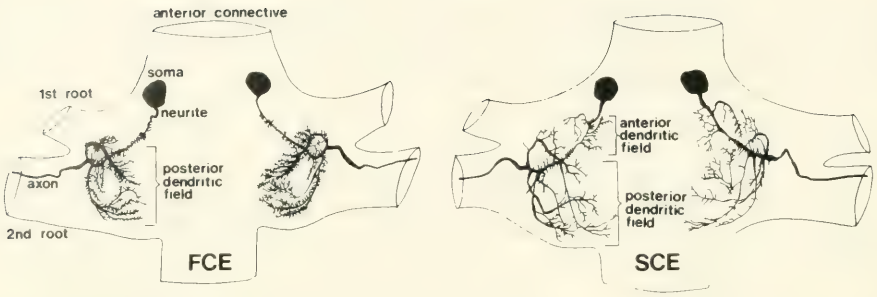


FIGURE 10. Camera lucida drawings of cobalt-filled motoneurons of paired FCE and SCE motoneurons in juvenile lobsters with cutter (right side) and crusher (left side) claws. Magnification, 40×. (from Hill and Govind, 1983).

arise from the neurite and are restricted to their respective hemiganglia, differ between FCE and SCE neurons. The SCE has a much more elaborate dendritic field than the FCE. Thus, whereas the FCE has only a distal dendritic field of two primary branches, the SCE has a distal field of several primary branches and a proximal field as well. In view of the striking asymmetry in behavior, external form and muscle composition of the paired claws, there was surprisingly no asymmetry between the homologous motoneurons.

A closer inhibitor (CI) axon is present in the 1st larval stage muscle (Fig. 9) (King and Govind, 1980) judging from the occurrence of neuromuscular terminals populated by ellipsoid-shaped synaptic vesicles which are characteristic of inhibitory terminals (Atwood *et al.*, 1972). Whether there is a single CI cannot be deduced from this type of morphological evidence. In the juvenile 4th stage, however, a single class of inhibitory junctional potentials (ijp) is seen with stimulation of the closer nerve suggesting the presence of a single CI (Costello *et al.*, 1981). The adult closer muscles receive a lone CI (Hill and Govind, 1981) which is seen to be shared with several other cheliped muscles (Hill and Lang, 1979).

Distribution. In the juvenile 4th, 5th, and 6th stages, the innervation pattern of the FCE and SCE axons is similar between the paired muscles (Table III) (Lang *et al.*, 1980; Costello *et al.*, 1981). The majority of fibers receive both axons while a small number receive each axon exclusively. In the adult the pattern is dramatically different between cutter and crusher muscles. Most of the fibers in the cutter receive

TABLE III

Distribution of innervation by FCE and SCE axons in claw closer muscle of juvenile lobsters where the pattern is similar between the paired claws and in adult lobsters where the pattern differs between the paired (cutter and crusher) claws (from Costello et al., *1981).*

	% innervation		
	FCE	SCE	FCE + SCE
Stage 4	9	5	86
Stage 5	18	5	77
Stage 6	9	9	82
Adult cutter	64	16	20
Adult crusher	15	18	67

FCE only, a few receive either the SCE only or both SCE and FCE together. In contrast, most of the crusher fibers receive both axons, while a few receive either FCE or SCE exclusively. This signifies a clear change in the innervation patterns between juvenile and adult muscles. Since the paired juvenile muscles are symmetrical in their innervation they may be regarded as being undifferentiated compared to the adult cutter and crusher muscles which have their own peculiar innervation pattern representing the differentiated condition. From the undifferentiated juvenile state where the large majority (80%) of fibers received both FCE and SCE axons, selective elimination of SCE would result in the adult distribution of 64% FCE innervation in the cutter. Synaptic elimination, on a smaller scale, of the FCE elements would give rise to the adult value of 16% SCE innervation. Similar processes would operate in finalizing the innervation to the crusher muscle where synapse elimination would affect fewer fibers since only a small number are supplied exclusively by each axon. According to this scheme the final pattern of innervation is refined by selective elimination of cutter FCE or SCE synapses from an initial (juvenile) condition where both axons are present. There may well be alternative methods for achieving the adult innervation patterns, such as the generation of new synapses, though the proposed mechanism is the most parsimonious one.

The distribution of the CI axon has not been mapped out for either developing (juvenile) or adult lobsters. In the few instances where CI has been detected, it was found on fibers with SCE innervation (Costello *et al.*, 1981; Hill and Govind, 1982).

Synaptic properties. In adult lobsters the neuromuscular synapses provided by the FCE and SCE axons differ in their physiological and fine structural properties. Thus the amplitude of the excitatory junctional potential (ejp) at 1 Hz stimulation is generally larger for the FCE than for the SCE synapses (Fig. 11) (Govind and Lang, 1974; Costello *et al.*, 1981). Conversely the degree of facilitation of the ejps calculated as the ratio of the ejp amplitude at 10 and 1 Hz is greater for the SCE than the FCE synapses. The SCE synapses were more fatigue-resistant and showed better recovery following fatigue than their FCE counterparts. FCE fine structure is relatively simple in having small-diameter terminals each with few synaptic vesicles, a single long synapse and little if any postsynaptic apparatus (Hill and Govind, 1981). The SCE innervation is more complex in having a wide size range of terminals each with many synaptic vesicles, several short synapses, and an extensive postsynaptic apparatus. The above data shows a clear distinction between neuromuscular synapses of the FCE and SCE axons.

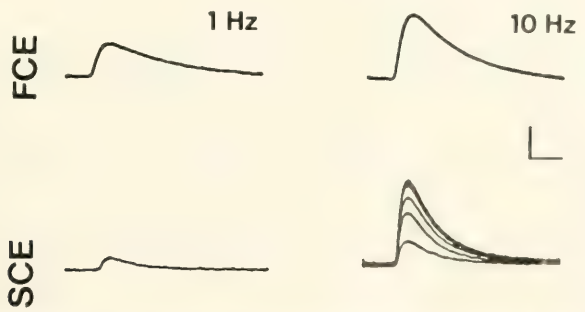


FIGURE 11. Synaptic properties represented by the amplitude of the ejp at 1 Hz stimulation and its degree of facilitation at 10 Hz for the FCE and SCE axons in an adult cutter closer muscles. Vertical calibration, FCE, 5 mV; SCE 4 mV. Horizontal calibration, 20 ms. (Costello *et al.*, 1981).

The physiological data reveals no differences in homologous synapses between cutter and crusher claws except perhaps that the FCE synapses show a greater maximal EJP amplitude in the cutter compared to the crusher claw. The other difference between the paired claws is that synapses of both axons tended to be more fatigue-resistant in the crusher compared to the cutter claw (Govind and Lang, 1974).

The development of neuromuscular synapses from the excitatory axons has been studied using electrophysiology and electron microscopy. In a few recordings made from the larval 2nd stage muscle, large ejps of approximately 10 and 20 mV were characteristic of putative SCE and FCE synapses respectively (Hill and Govind, 1984). These large synaptic potentials of the FCE axon often produced secondary regenerative responses. In a larger sampling of synapses from the larval 3rd stage the mean ejp size was 6 mV with a range of 4 to 10 mV for the FCE synapses. The SCE synapses were considerably smaller with a mean of 3 mV and a range of 2 to 4 mV. The amount of facilitation was similar for the two types of synapses. However, the FCE synapses often produced regenerative responses and displayed fewer transmission failures than the SCE synapses. In the juvenile 4th, 5th, and 6th stages (Costello *et al.*, 1981) ejps of the FCE displayed a wide range in amplitude though the mean size was similar to the larval and adult forms signifying that they had reached their final condition. On the other hand, ejps of the SCE axon in these juvenile stages had as narrow a range of amplitudes as those in the larval stage. The wider spread in ejp amplitude typical of the adult SCE synapses must presumably come with maturation. The other difference between FCE and SCE synapses in the juvenile lobsters is the fact that the former synapses are much more fatigue-resistant than the latter; a situation which is exactly the reverse of that found in adult lobsters.

In terms of the size of the ejp, synapses differentiate into FCE and SCE types early in the larval stages while the properties of facilitation and fatigue-sensitivity mature later during juvenile development.

Structural aspects of the development of excitatory synapses were examined by serial section electron microscopy of the closer muscle in a larval 1st stage, a juvenile 4th stage, and an adult lobster (King and Govind, 1980). No attempt was made to determine whether the terminals belonged to the FCE or SCE axons. There was a tremendous proliferation of excitatory innervation from the 1st larval stage where it was restricted to four discrete locations over the entire muscle to individual muscle fibers in the adult. Concomitantly there is a ten-fold and significant increase in the mean size of synapses between larval and adult stages (Table IV). The mean size of terminals varied considerably among the three stages examined and showed no consistent trend. On the other hand, the presynaptic dense bars, representing active sites of transmitter release were consistently similar in size and were found in the majority (>60%) of synapses. Synaptic development therefore consists of an increase in number and size of excitatory synapses which occur in tandem with the increase in mass of the closer muscle. From within this overall pattern of synaptic development, there is a need to distinguish between FCE and SCE synapses in order to understand how their final distribution within the closer muscle forms. A start has been made in this direction by examining physiologically identified FCE and SCE terminals in juvenile lobsters (Hill and Govind, 1981). The FCE innervation is relatively simple, consisting of small terminals each with a single synapse, few synaptic vesicles and limited postsynaptic apparatus. In contrast the SCE innervation is more complex, having larger and more variable terminals each with several short synapses, many synaptic vesicles, and an extensive postsynaptic apparatus.

Firing patterns. The *in vivo* activity of the FCE and SCE axons during reflex closing of the claws was analyzed in one to three-year-old juvenile lobsters with

TABLE IV

Quantitative comparison of excitatory nerve terminals, synapses, and presynaptic dense bars in the claw closer muscle of a larval (1st stage), juvenile (4th stage), and adult lobster (from King and Govind, 1980)

	1st stage	4th stage	Adult
Nerve terminals:			
Length of muscle fiber serially sectioned (μm)	10.23	11.12	12.05
Total number	6	5	5
Mean surface area (μm^2)	34.63	53.73	19.49
($\bar{x} \pm$ S.E.M.)	±10.93	±32.93	±10.55
Synapses:			
Number completely sectioned	29	51	15
Mean number per terminal	4.83	10.20	3.20
($\bar{x} \pm$ S.E.M.)	±1.79	±5.04	±0.89
Mean surface area (μm^2)	0.079	0.136	0.805
($\bar{x} \pm$ S.E.M.)	±0.010	±0.012	±0.174
Presynaptic dense bars:			
Total number	22	40	13
Mean surface area (μm^2)	0.018	0.016	0.017
($\bar{x} \pm$ S.E.M.)	±0.005	±0.002	±0.002
Mean number per synapse	0.759	0.784	0.867
($\bar{x} \pm$ S.E.M.)	±0.128	±0.081	±0.236

dimorphic claws (Costello *et al.*, 1984). While the dactyl was free to move, the rest of the claw and the animal was immobilized in order to permit recordings of ejps from the closer muscle fibers. Under these conditions, the FCE fired only during rapid closing at a lower frequency and duration than the SCE which fired only during slow closing of the cutter claw (Table V). However, the crusher FCE and SCE axons were active during fast and slow closing respectively and their firing patterns were similar. This similarity was also found between the two axons during maintained closing of the crusher. Thus a clear distinction is found between FCE and SCE axons in the cutter but not in the crusher claw.

When the homologous motoneurons are compared, the FCE of the crusher has a significantly higher firing frequency and burst duration than its cutter counterpart during fast closing of the claw (Table V). The homologous SCEs, however, displayed

TABLE V

In vivo *firing frequency and burst duration of FCE and SCE axons during fast, slow, and maintained closing of cutter and crusher claws of intact lobsters (from Costello* et al., *1984)*

Claw type	Closing behavior	Motoneuron type	Frequency (Hz) $\bar{x} \pm$ S.D.	Burst duration (ms) $\bar{x} \pm$ S.D.	n
Cutter	fast	FCE	2 ± 2	55 ± 26	46
Cutter	slow	SCE	37 ± 24	361 ± 143	51
Cutter	maintained	SCE	15 ± 9	1963 ± 1351	31
Crusher	fast	FCE	37 ± 27	215 ± 94	44
Crusher	maintained	FCE	10 ± 9	1010 ± 630	13
Crusher	slow	SCE	31 ± 12	406 ± 175	17
Crusher	maintained	SCE	18 ± 13	1278 ± 707	23

a close similarity in their firing patterns during slow closing. Maintaining the closed claw was achieved by the FCE and SCE axons in the crusher but only by the SCE in the cutter claw though all three axons were similar in their firing patterns. Thus the FCE alone showed an asymmetry in firing patterns between cutter and crusher claws in intact juvenile lobsters.

In contrast to the above, the *in vitro* activity of the adult motoneurons shows a clear asymmetry between FCE and SCE in *both* claws and between *both* homologs (Govind and Lang, 1981). Activity of the motoneurons was recorded from their respective somata in response to electrical stimulation of the mixed nerve roots in an isolated claw-ganglion preparation. In both claws, the FCE fired at a lower frequency and for a shorter time than the SCE. When homologous somata were examined, the crusher FCE and SCE produced higher frequencies and longer bursts of spikes than their cutter counterparts (Fig. 12). Since this asymmetry was found in response to both sensory stimulation via the 2nd nerve root and depolarization of the soma it could have both an extrinsic (sensory) and intrinsic (built-in) origin.

The motoneurons also produce a distinct pattern of paired impulses (Costello *et al.*, 1981; Govind and Hill, 1982) which are functionally more effective in generating muscle tension than uniformly spaced impulses of the same average frequency (Ripley and Wiersma, 1953; Govind and Lang, 1974). In intact juvenile lobsters, paired impulses with interpulse intervals of between 8 to 13 ms, were found for both FCE and SCE axons in both claws. The only indication of asymmetry in this firing pattern

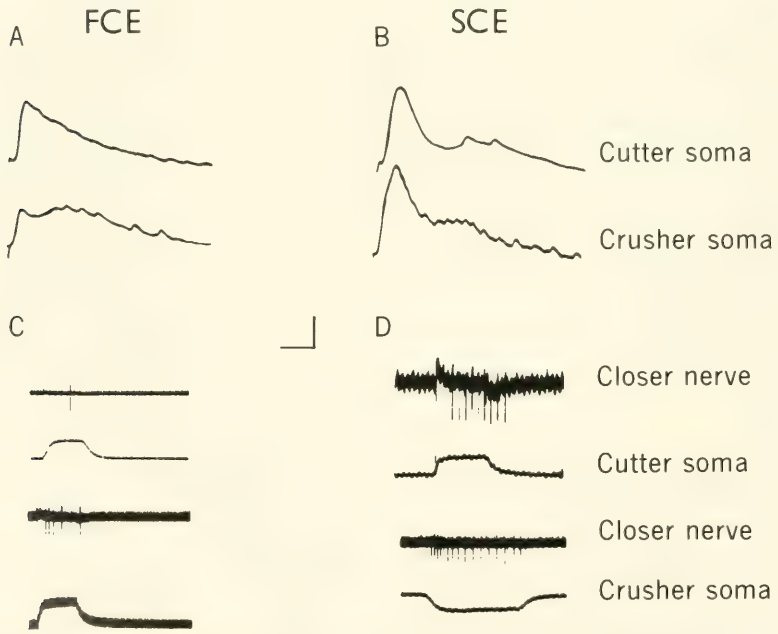


FIGURE 12. Firing patterns of homologous FCE and SCE neurons recorded either from their somata (A, B) in response to sensory stimulation via the 2nd nerve root or from the closer nerve (C, D) in response to depolarization of their somata. For each homologous pair, the crusher motoneuron shows a greater response than its cutter counterpart. Vertical calibration, 4 mV in A; 10 mV in B; 1 μA in C lower trace; 2 μA in C upper trace and D. Horizontal calibration, 40 ms in A, B, D lower trace; 100 ms in C, D upper trace (from Govind and Lang, 1981).

was for the homologous FCE axon which produced paired impulses almost all the time in the crusher but only 25% of the time in the cutter. The homologous SCE axons resembled each other in producing paired impulses 60% of the time. In isolated claw-ganglion preparations of adult lobsters, depolarization of the soma gave rise to paired impulses in FCE and SCE motoneurons thereby strongly implicating an endogenous mechanism for the generation of this patterned activity.

The development of the characteristic firing patterns for the FCE motoneurons alone has been examined in intact juveniles (Costello and Govind, 1983b; unpub.) where closing has been reflexly evoked. In the juvenile 4th and 5th stage the homologous FCE fire at a similar frequency of 100–150 Hz for 150–200 ms. In the subsequent juvenile stages till about the 12th stage, both the frequency and duration of firing decreases dramatically in the putative cutter claw to <10 Hz for <50 ms which approximates the adult condition. In the putative crusher the duration fluctuates between 100–200 ms while the frequency gradually decreases to <50 Hz which is reminiscent of the adult condition. The activity patterns of the homologous FCE have essentially matured by the time the lobster is a year old.

The closer muscles have a timetable similar to that of the FCEs in achieving their final composition of fiber types. Whether there is any causal relationship between the development of asymmetry in the firing patterns of the homologous FCE motoneurons and in the fiber composition of the closer muscles cannot be deduced from this correlation. However, transformation of fast fibers to slow and the resultant differentiation of a crusher muscle with all slow fibers can be prevented by denervation or tenotomy in the early juvenile stages (Govind, 1981; Govind and Kent, 1982). Since these treatments reduce or eliminate active muscle tension mediated by motor impulses, they implicate the motoneurons in directing the differentiation of muscle fiber types. On the other hand, these experiments also suggest that it may be the overall level of active muscle tension which transforms fast fibers to slow. Consequently, the trigger for muscle transformation may well reside in the level of motoneuronal activity of both excitors, FCE and SCE, and the inhibitor, CI, axons. The claw muscle experiencing the higher level of motor activity would become the crusher while its counterpart muscle would become the cutter.

Motoneurons to the opener muscle

The innervation to this muscle in lobsters has received scant attention compared to the very extensive studies of the homologous muscle in crayfish (reviewed by Atwood, 1976). The discovery of subtle asymmetries in the neuromuscular system in the opener muscle between cutter and crusher claws (Govind *et al.*, 1981) has initiated a more detailed current investigation (G. Kass-Simon and K. Mearow, unpub.) which provides the basis for most of the comments given here unless otherwise acknowledged.

Number and type. The opener muscle in the limbs of decapod crustaceans is supplied by an excitor (OE) and inhibitor (OI) motoneuron (Wiersma, 1961). While the OE also innervates the stretcher muscle in the next proximal segment, the OI is a private motoneuron. However, more recent evidence suggests that the CI also innervates the opener muscle (T. J. Wiens, pers. comm.). The OE is reminiscent of a slow excitor axon as it does not cause rapid opening of the dactyl and is more fatigue-resistant. The development of excitatory and inhibitory innervation has not been examined in the lobster though both types of synapses are present immediately after hatching in the homologous opener muscle in the crayfish (Atwood and Kwan, 1976).

Distribution. Being the only excitor axon, OE may be expected to innervate all fibers of the adult opener muscle. The presence of OI and CI however has not been detected in all fibers examined suggesting a regional distribution of innervation for each of these two axons which may account for the fact that they were not recognized as separate axons in the past.

Synaptic properties. Generally the amplitude of the ejps are small ranging from <1 mV to 5 mV; many being visible only after a bout of high frequency stimulation designed to produce facilitation and summation. All of the excitatory synapses showed moderate to strong facilitation with repeated stimulation. The OI synapses also gave small junctional potentials which were either hyperpolarizing or depolarizing in sign. These ejps exerted considerable postsynaptic inhibition judging from the fact that they reduced the size of the ejp considerably. Almost complete elimination of the ejp occurred occasionally suggesting pre-synaptic inhibition similar to that found in the homologous motoneurons in the crayfish opener muscle. In terms of their physiology the OE and OI synapses in lobster resemble their counterparts in crayfish (Atwood and Bittner, 1971). Consequently they may also resemble them in ultrastructure which has been extensively described in crayfish (Jahromi and Atwood, 1974).

Firing patterns. The crusher OE has a higher frequency of firing and longer burst duration than its cutter counterpart in response to nerve root stimulation in isolated ganglia of adult lobsters (Govind *et al.,* 1981). The crusher OE is also more resistant to fatigue when stimulated repetitively than the cutter OE. This asymmetry in firing patterns between homologous OE motoneurons *in vitro* forebodes a similar asymmetry in the intact lobster. The ontogeny of these firing patterns is unknown.

Sensory neurons

In a singular attempt to document asymmetry in the sensory system between paired cutter and crusher claws, the number and size of axons was determined in the nerve roots to a juvenile lobster (Govind and Pearce, 1984). The nerve roots are mixed, containing both sensory and motor axons. However, since the motor axons are bilaterally constant and relatively few in number, the majority of axons in the nerve roots are sensory. The total numbers of axons in the first root were approximately 16,000 for the crusher and 13,000 for the cutter; which gave a crusher-cutter ratio of 1.22. For the second root the counts were 119,000 for the crusher and 124,000 for the cutter which gave a ratio of 0.96. The slight asymmetries in the roots proved not to be significant in random samples from homologous regions. Furthermore, a representative sampling of the axon diameters showed a parallel distribution in all size classes between crusher and cutter claws. Consequently, there does not appear to be a asymmetry in the numbers and sizes of sensory axons between the paired claws in a juvenile lobster. However, in adults the external dimorphism between the paired claws is much more pronounced than in the juvenile and there is the possibility that the sensory system may be asymmetric.

Similarly, no differences in the distribution of four different types of cuticular hair organs were detected between cutter and crusher claws of "subadult" lobsters (Solon and Cobb, 1980). These four cuticular hair organs which are regarded as mechanosensory in function differ basically in the length of the sensilla: type I are the longest (70–130 μm), type II slightly smaller at 30–60 μm, type III still smaller but located in a raised protuberance, and type IV are simply 1 μm long conical hairs occurring in clusters. Types II and III differed in distribution between dorsal and ventral sides and among different areas of the claw. More interesting was their dis-

tribution in a juvenile lobster with symmetrical claws. Type IV receptors were just as ubiquitous in the juvenile as in the subadult. Type III receptors, however, had a lower density in the juvenile than in the subadult denoting the addition of these hair organs during growth of the claws. On the other hand, types I and II with a higher density in the juvenile than in the subadults are apparently not added during growth. These differences in density of particular types of hair organs between juveniles and subadults may reflect changes in behavior during development which have been documented previously (Lang *et al.*, 1977c).

Comparison with other Asymmetric Systems

Claw asymmetry is not uncommon among crustaceans and it may be instructive to review how it arises during development in fiddler crabs and how it is maintained during regeneration in snapping shrimps. Adult male fiddler crabs have a hypertrophied major claw used for courtship and defence and a minor claw used for feeding and grooming (reviewed by Crane, 1977). The asymmetry in external form is matched by an asymmetry in muscle mass (Rhodes, 1977), soma size, and dendritic field of the motoneurons (Young and Govind, 1983), and in the numbers of sensory axons (Govind and Pearce, 1984). Early in development the paired claws are symmetrical. The asymmetry develops following the loss of one of the paired claws during a critical period which extends from the megalopa to a young crab stage (Morgan, 1923, 1924; Yamaguchi, 1977). If both claws are removed at this stage then no major claw develops; if both are kept intact during this critical period, then paired major claws develop. Consequently the loss of a claw during development triggers the remaining one to differentiate into a major claw in male fiddler crabs. Once the claw asymmetry is established it remains fixed and removal of either major or minor claw will cause the same type to regenerate. This is similar to the situation in lobsters but unlike that in snapping shrimps where claw laterality is not fixed in the adult.

The major or snapper claw in Alpheid shrimps is used in defence when it ejects a jet of water on closing and at the same time makes a loud popping sound; the minor or pincer claw is used for feeding and grooming. In adult shrimps, autotomy of the snapper results in the regeneration of a pincer in its place while the existing pincer transforms into a snapper (Prizbram, 1901; Wilson, 1903). This pincer to snapper transformation involves several changes: a hypertrophy and differentiation in the external form, a hypertrophy of the motoneuron somata to the closer muscle (Mellon *et al.*, 1981), a hypertrophy of the closer muscle and the transformation of its fast fibers to slow, and an increase in facilitation of the excitatory neuromuscular synapses (Stephens and Mellon, 1979). Transformation is either prevented if the nerve to the pincer is transected at the time of snapper removal (Wilson, 1903) or promoted if the nerve to the snapper alone is transected (Mellon and Stephens, 1978). The pincer can be regarded as an undifferentiated snapper which is arrested in its development by the existing contralateral snapper. Once this inhibition is removed by autotomy of the snapper or transection of its nerve, the pincer completes its differentiation to a snapper and at the same time arrests the development of the newly regenerating claw to a pincer type. Clearly, the maintenance of claw asymmetry and its reversal in adult snapping shrimps is under neural control (Mellon, 1981). This is similar to how claw type is determined in juvenile lobsters (Govind, 1981) where denervation of one claw causes the contralateral one to become the crusher (Govind and Kent, 1982). However, in lobsters, once claw asymmetry is determined during juvenile development, it remains fixed throughout adult life whereas in snapping shrimps it can be altered in the adult.

Future Prospects

One aspect of our fascination with asymmetric systems, whether it be cerebral dominance in humans or claw lateralization in lobsters, lies in being able to understand how it arises from a bilaterally symmetrical body plan. Such a goal is feasible in the neuromuscular system of the lobster claw because certain features of this system are of advantage in studying development. First the lobster has a protracted period of development, consisting of a 9–11 month embryonic period, a two-week larval period, and a 5–7 year juvenile period (Herrick, 1895, Hughes *et al.*, 1972) which is divided into discrete stages by the molt cycle. All these stages can be reared in the laboratory (Hughes *et al.*, 1974; Lang, 1975). Second, there are only two muscles, the antagonistic opener and closer, which make up the claw. Third, each muscle is innervated by few motoneurons: two excitors and an inhibitor in the closer and a single excitor and two inhibitors in the opener (Wiersma, 1961; T. J. Wiens, pers. comm.). Fourth, and perhaps most important of all, is the fact that claw laterality is determined during a critical two-week period of juvenile development, between the 4th and 5th stages, when the claws can be experimentally manipulated (Emmel, 1908; Lang *et al.*, 1978).

Indeed, manipulations such as tenotomy of the opener or closer muscle, or denervation can suppress the differentiation of a crusher claw, resulting in lobsters with paired cutter claws (Govind and Kent, 1982). In terms of the fiber composition of the closer muscle, this means that fast fibers are prevented from transforming to slow because of a lack of nerve-mediated muscle tension. Since there are only three motoneurons to the closer muscle, each uniquely identifiable, it is possible to examine the influence of each on muscle development. Experimental manipulations of these motoneurons such as selective deletion or electrical stimulation during the critical juvenile period, should pinpoint the role of motoneurons in the differentiation of muscle fiber types.

The experiments proposed above would test the hypothesis that it is the difference in motor output in the paired claws that determines laterality. The claw receiving the greater overall motor output during the critical developmental period transforms its fast fibers to slow and becomes the crusher muscle with all slow fibers. In the absence of a certain level of motor output this transformation is prevented and the closer muscle remains with predominantly fast fibers (Lang *et al.*, 1978) characteristic of the cutter muscle which is presumably the primitive condition. As a corollary, by controlling the motor output to the juvenile undifferentiated muscle we should be able to produce a crusher not only on a prescribed side but on both sides. These experiments, currently in progress, would explain how the asymmetry in muscle fiber composition arises during development.

There is still a need to explain the asymmetry in firing patterns of the homologous motoneurons, specifically, to what extent are they due to the intrinsic (cable) properties of the motoneurons or to extrinsic (synaptic) influences. This will necessitate examining the electrical properties of the homologous motoneurons in the juvenile stages and their synaptic input. If the asymmetry in firing patterns is influenced by the synaptic input we would need to explore its nature and number. This involves primarily the sensory system of the claws though ascending and descending inputs within the ganglion can also influence the motoneuron firing patterns.

Apart from the above experiments revolving around the sensory system and ganglion there is the need to explain the differences in the distribution of the homologous motoneurons onto the closer muscles. From the juvenile condition where the majority of fibers in both muscles are innervated by both excitatory axons, the cutter closer muscle has predominantly FCE innervation while the crusher muscle has predomi-

nantly mixed, FCE and SCE, innervation (Costello *et al.*, 1981). Can this asymmetry in the pattern of synaptic connections be explained by the selective elimination of synapses in the developing cutter muscle, as has been seen in a lobster abdominal muscle (Stephens and Govind, 1981). An equally challenging task would be to understand why such an asymmetry arises: is it due to competition between the motoneurons or is it influenced by the muscle fiber properties?

Finally, a significant component missing from the present consideration of the claw neuromuscular system is the inhibitory (CI) motoneuron. There is a clear need to examine both its central and peripheral mechanisms in order to establish its role in claw asymmetry and to follow its development.

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LITERATURE CITED

Atwood, H. L. 1967. Crustacean neuromuscular mechanisms. *Am. Zool.* **7**: 527–551.

Atwood, H. L. 1973. An attempt to account for the diversity of crustacean muscles. *Am. Zool.* **13**: 357–378.

Atwood, H. L. 1976. Organization and synaptic physiology of crustacean neuromuscular systems. *Prog. Neurobiol.* **7**: 291–391.

Atwood, H. L., and G. D. Bittner. 1971. Matching of excitatory and inhibitory inputs to crustacean muscle fibers. *J. Neurophysiol.* **34**: 157–170.

Atwood, H. L., and I. Kwan. 1976. Development of synapses in crayfish opener muscle. *J. Neurobiol.* **7**: 289–312.

Atwood, H. L., F. Lang, and W. A. Morin. 1972. Synaptic vesicles: selective depletion in crayfish excitatory and inhibitory axons. *Science* **176**: 1353–1355.

Bittner, G. D., 1968. The differentiation of crayfish muscle fibers during development. *J. Exp. Zool.* **167**: 439–456.

Bittner, G. D., and D. L. Traut, 1978. Growth of crustacean muscle and muscle fibers. *J. Comp. Physiol.* **124**: 277–285.

Burke, R. E. 1981. Motor units: anatomy, physiology and functional organization. Pp. 345–422 in *Handbook of Physiology. The Nervous System,* Vol. II, J. M. Brookhart and V. B Mountcastle, eds. Williams & Williams Co., Baltimore.

Corballis, M. C., and M. J. Morgan. 1978. On the biological basis of human laterality: evidence for a maturational left-right gradient. *Behav. Brain Sci.* **2**: 261–336.

Costello, W. J., and C. K. Govind. 1983a. Contractile responses of single fibers in lobster claw closer muscles: correlation with structure, histochemistry and innervation. *J. Exp. Zool.* **227**: 381–393.

Costello, W. J., and C. K. Govind. 1983b. Correlation of motoneuron activity, muscle proteins and claw dimorphism in the lobster *Homarus americanus*. *Am. Zool.* **23**: 902.

Costello, W. J., and C. K. Govind. 1984. Contractile proteins of fast and slow fibers during differentiation of lobster claw closer muscles. *Dev. Biol.* (In Press)

Costello, W. J., and F. Lang. 1979. Development of the dimorphic claw closer muscles of the lobster, *Homarus americanus*. IV. Changes in functional morphology during growth. *Biol. Bull.* **156**: 179–195.

Costello, W. J., R. Hill, and F. Lang. 1981. Innervation patterns of fast and slow motor neurones during development of a lobster neuromuscular system. *J. Exp. Biol.* **91**: 271–284.

Costello, W. J., R. Hill, and F. Lang, 1984. Firing patterns of closer motoneurons during reflex activity in the dimorphic claws of the lobster. *J. Exp. Zool.* (In Press)

Crane, J., 1977. *Fiddler crabs of the world. Ocypodidae: Genus. Uca.* Princeton University Press, Princeton, New Jersey. 736 pp.

El-Haj, A. J., C. K. Govind, and D. F. Houlihan. 1984. Growth of lobster leg muscle fibers over intermolt and molt. *J. Crust. Biol.* **4**: 536–545

Emmel, V. E. 1908. The experimental control of asymmetry at different stages in the development of the lobster. *J. Exp. Zool.* **5**: 471–484.

GOUDEY, L. R., AND F. LANG. 1974. Growth of crustacean muscle: asymmetric development of the claw closer muscles in the lobster, *Homarus americanus*. *J. Exp. Zool.* **189:** 421–427.

GOVIND, C. K., 1981. Does exercise influence the differentiation of lobster muscles. Pp. 215–253 in *Locomotion and Energetics in Arthropods,* C. F. Herreid II and C. R. Fourtner, eds., Plenum Publ. Corp., New York.

GOVIND, C. K., AND H. L. ATWOOD. 1982. Organization of neuromuscular systems. Pp. 63–103 in *The Biology of Crustacea,* Vol. 3, *Neurobiology: Structure and Function,* D. E. Bliss, H. L. Atwood, and D. C. Sandeman, eds. Academic Press, New York.

GOVIND, C. K., AND R. H. HILL. 1982. Paired impulses in lobster claw motoneurons: *in vitro* and *in vivo* production. *Can. J. Zool.* **60:** 1096–1099.

GOVIND, C. K., AND K. S. KENT. 1982. Transformation of fast fibers to slow prevented by lack of activity in developing lobster muscle. *Nature* **298:** 755–757.

GOVIND, C. K., AND F. LANG. 1974. Neuromuscular analysis of closing in the dimorphic claws of the lobster *Homarus americanus*. *J. Exp. Zool.* **190:** 281–288.

GOVIND, C. K., AND F. LANG. 1978. Development of the dimorphic claw closer muscles of the lobster, *Homarus americanus*. III. Transformation to dimorphic muscles in juveniles. *Biol. Bull.* **154:** 55–67.

GOVIND, C. K., AND F. LANG. 1979. Physiological asymmetry in the bilateral crusher claws of a lobster. *J. Exp. Zool.* **297:** 27–32.

GOVIND, C. K., AND F. LANG. 1981. Physiological identification and asymmetry of lobster claw closer motoneurones. *J. Exp. Biol.* **94:** 329–339.

GOVIND, C. K., AND J. PEARCE. 1984. Lateralization in number and size of sensory axons to the dimorphic chelipeds of crustaceans. (Submitted)

GOVIND, C. K., H. L. ATWOOD, AND F. LANG. 1974. Sarcomere length increases in developing crustacean muscle. *J. Exp. Zool.* **189:** 395–400.

GOVIND, C. K., J. SHE, AND F. LANG. 1977. Lengthening of lobster muscle fibers by two age-dependent mechanisms. *Experientia* **33:** 35–36.

GOVIND, C. K., P. J. STEPHENS, AND V. TRINKAUS-RANDALL. 1981. Differences in motor output and fiber composition of the opener muscle in lobster dimorphic claws. *J. Exp. Zool.* **218:** 363–370.

GUTH, L. 1968. Trophic influences of nerve on muscle. *Physiol. Rev.* **48:** 645–687.

GUTMANN, E., 1976. Neurotrophic relations. *Ann. Rev. Physiol.* **38:** 177–216.

HAJEK, I., N. CHARI, A. BASS, AND E. GUTMANN. 1973. Differences in contractile and some biochemical properties between fast and slow abdominal muscles of the crayfish (*Astacus leptodactylus*). *Physiol. Bohemslov.* **22:** 603–612.

HARRIS, A. J. 1974. Inductive functions of the nervous system. *Ann. Rev. Physiol.* **36:** 251–305.

HERRICK, F. H. 1895. The American lobster: a study of its habits and development. *Fish Bull. U. S.* **15:** 1–252.

HERRICK, F. H. 1911. Natural history of the American lobster. *U. S. Bur. Fish.* **29:** 149–408.

HILL, R. H., AND C. K. GOVIND. 1981. Comparison of fast and slow synaptic termials in lobster muscle. *Cell Tiss. Res.* **221:** 303–310.

HILL, R. H., AND C. K. GOVIND. 1982. Functional subdivision within a lobster motor unit. *Experientia* **38:** 362–363.

HILL, R. H., AND C. K. GOVIND. 1983. Fast and slow motoneurons with unique forms and activity patterns in lobster claws. *J. Comp. Neurol.* **218:** 327–333.

HILL, R. H., AND C. K. GOVIND. 1984. Larval innervation of lobster claw closer muscle. *J. Exp. Zool.* **229:** 393–399.

HILL, R. H., AND F. LANG. 1979. A revision of the inhibitory innervation pattern of the thoracic limbs of crayfish and lobster. *J. Exp. Zool.* **208:** 129–135.

HUGHES, J. T., J. J. SULLIVAN, AND R. SHLESER. 1972. Enhancement of lobster growth. *Science* **177:** 1110–1111.

HUGHES, J. T., R. A. SHLESER, AND G. TCHOBANOGLOUS. 1974. A rearing tank for lobster larvae and other aquatic species. *Prog. Fish. Cult.* **36:** 129–132.

JAHROMI, S. S., AND H. L. ATWOOD. 1971. Structural and contractile properties of lobster leg muscle fibers. *J. Exp. Zool.* **176:** 475–486.

JAHROMI, S. S., AND H. L. ATWOOD. 1974. Three dimensional ultrastructure of the crayfish neuromuscular apparatus. *J. Cell Biol.* **63:** 599–613.

JAHROMI, S. S., AND M. P. CHARLTON, 1978. Transverse sarcomere splitting: a possible means of longitudinal growth in crab muscle. *J. Cell Biol.* **80:** 736–742.

JOLESZ, F., AND F. A. SRETER. 1981. Development, innervation, and activity-pattern induced changes in skeletal muscle. *Ann. Rev. Physiol.* **43:** 531–552.

KENT, K. S., AND C. K. GOVIND. 1981. Two types of tonic fibers in lobster muscle based on enzyme histochemistry. *J. Exp. Zool.* **215:** 113–116.

KING, J. J., AND C. K. GOVIND. 1980. Development of excitatory innervation in lobster claw closer muscle. *J. Comp. Neurol.* **194:** 57–70.

LANG, F. 1975. A simple culture system for juvenile lobsters. *Aquaculture* **6:** 389–393.

LANG, F., W. J. COSTELLO, AND C. K. GOVIND. 1977a. Development of the dimorphic claw closer muscles of the lobster, *Homarus americanus*. I. Distribution of fiber types in adults. *Biol. Bull.* **152:** 75–83.

LANG, F., C. K. GOVIND, AND J. SHE. 1977b. Development of the dimorphic claw closer muscles of the lobster, *Homarus americanus*. II. Distribution of muscle fiber types in larval forms. *Biol. Bull.* **152:** 382–391.

LANG, F., C. K. GOVIND, W. J. COSTELLO, AND S. I. GREENE. 1977c. Developmental neuroethology: changes in escape and defensive behavior during growth of the lobster. *Science* **197:** 682–685.

LANG, F., C. K. GOVIND, AND W. J. COSTELLO. 1978. Experimental transformation of muscle fiber properties in lobster. *Science* **201:** 1037–1039.

LANG, F., M. M. OGONOWSKI, W. J. COSTELLO, R. HILL, B. ROEHRIG, K. KENT, AND J. SELLERS. 1980. Neurotrophic influence on lobster skeletal muscle. *Science* **207:** 325–327.

LEHMAN, W., AND A. G. SZENT-GYÖRGYI. 1975. Regulation of muscular contraction: distribution of actin control and myosin control in the animal kingdom. *J. Gen. Physiol.* **66:** 1–30.

MELLON, DEF., JR. 1981. Nerves and the transformation of claw type in snapping shrimp. *Trends Neurosci.* **4:** 245–248.

MELLON, DEF., JR., AND P. J. STEPHENS. 1978. Limb morphology and function are transformed by contralateral nerve section in snapping shrimps. *Nature* **272:** 246–248.

MELLON, DEF., JR., J. A. WILSON, AND C. E. PHILLIPS. 1981. Modification of motoneuron size and position in the central nervous system of adult snapping shrimp. *Brain Res.* **223:** 134–140.

MORGAN, T. H. 1923. The development of asymmetry in the fiddler crab. *Am. Nat.* **57:** 269–273.

MORGAN, T. H. 1924. The artificial induction of symmetrical claws in male fiddler crabs. *Am. Nat.* **58:** 289–295.

NEAL, D. M., D. L. MACMILLAN, R. M. ROBERTSON, AND M. S. LAVERACK. 1976. The structure and function of the thoracic exopodites in the larvae of the lobster, *Homarus gammarus* L. *Phil. Trans. R. Soc. Lond. B.* **274:** 53–68.

NOTTEBOHM, F. 1977. Asymmetries in neural control of vocalization in the canary. Pp. 23–44 in *Lateralization in the Nervous System*, S. Harnard, R. W. Doty, L. Goldstein, J. Jaynes and G. Krauthamer, eds. Academic Press, New York.

OGONOWSKI, M. M., AND F. LANG. 1979. Histochemical evidence for enzyme differences in crustacean fast and slow muscle. *J. Exp. Zool.* **207:** 143–151.

OGONOWSKI, M. M., F. LANG, AND C. K. GOVIND. 1980. Histochemistry of lobster claw-closer muscles during development. *J. Exp. Zool.* **213:** 359–367.

PRZIBRAM, H. 1901. Experimentelle studien über Regeneration. *Arch. Entm. Mech. Org.* **11:** 321–345.

RHODES, W. R., JR. 1977. Anatomical and physiological correlates of asymmetry and courtship display by male fiddler crabs. Ph.D. Thesis, University of Wisconsin, Madison.

RIPLEY, S. H., AND C. A. G. WIERSMA. 1953. The effect of spaced stimulation of excitatory and inhibitory axons of the crayfish. *Physiol. Comp. Oecol.* **3:** 1–17.

SOLON, M. H., AND J. S. COBB. 1980. The external morphology and distribution of cuticular hair organs on the claws of the American lobster, *Homarus americanus* (Milne-Edwards). *J. Exp. Mar. Biol. Ecol.* **48:** 205–215.

STEPHENS, P. J., AND C. K. GOVIND. 1981. Peripheral innervation fields of single lobster motoneurons defined by synapse elimination during development. *Brain Res.* **212:** 476–480.

STEPHENS, P. J., AND DEF. MELLON, JR. 1979. Modification of structure and synaptic physiology in transformed shrimp muscle. *J. Comp. Physiol.* **132:** 97–108.

WIERSMA, C. A. G. 1955. An analysis of the functional differences between the contractions of the adductor muscles in the thoracic legs of the lobster *Homarus americanus*. *Arch. Neerl. Zool.* **11:** 1–13.

WIERSMA, C. A. G. 1961. The neuromuscular system. Pp. 191–240 in *The Physiology of Crustacea*, T. H. Waterman, ed. Academic Press, New York.

WILSON, E. B. 1903. Notes on the reversal of asymmetry in the regeneration of the chelae in *Alpheus hererochelis*. *Biol. Bull.* **4:** 197–210.

YAMAGUCHI, T. 1977. Studies on the handedness of the fiddler crab, *Uca lactea*. *Biol. Bull.* **152:** 424–436.

YOUNG, R. E., AND C. K. GOVIND. 1983. Neural asymmetry in male fiddler crabs. *Brain Res.* **280:** 251–262.

Reference: *Biol. Bull.* **167**: 120–123. (August, 1984)

APPARENT ABSENCE OF GAP JUNCTIONS IN TWO CLASSES OF CNIDARIA

G. O. MACKIE*, P. A. V. ANDERSON†, AND C. L. SINGLA*

† *C. V. Whitney Laboratory and Department of Physiology, University of Florida, St. Augustine, Florida 32086, and* **Biology Department, University of Victoria, Victoria, British Columbia, Canada V8W 2Y2*

ABSTRACT

Study of the literature and new observations by electron microscopy suggest that gap junctions are absent in the anthozoa and scyphozoa, but present in the hydrozoa. While this may help to explain the marked electrophysiological differences known to exist between the hydrozoa and the other two groups, it raises questions about how intercellular metabolic communication is achieved in the groups lacking gap junctions.

DISCUSSION

In many tissues of metazoans from *Hydra* to the mammals, cell interiors are directly linked by aqueous channels represented structurally by the channels of gap junctional particles, or connexons (Unwin and Zamphighi, 1980). The diameter of the channel, determined by probing with fluorescent molecules, is estimated to be 16–20 Å in mammals and 20–30 Å in insects (Schwarzmann *et al.*, 1981). Gap junctions are widely believed to be responsible for electrical and dye coupling and for the transmission of electrical signals within various excitable tissues. While final proof is still lacking, gap junctions probably play an important role in tissue homeostasis by allowing permeant molecules to equilibrate throughout groups of coupled cells, in transport of nutrients from cell to cell, and in the dissemination of regulatory molecules (reviewed by Loewenstein, 1981). These regulatory functions are thought to be especially important in embryonic and differentiating tissues where gap junctions are frequently found, along with electrical coupling.

Despite the circumstantial nature of much of the evidence for metabolic cooperation in cells joined by gap junctions, there can be little question that the first appearance of gap junctions in early metazoans represented a major organizational advance. The fact that sponges remain at the cellular rather than the tissue level (Hyman, 1940) may be due in large part to their apparent "genetic incapacity to produce gap junctions" (Mackie, 1984).

The lowest metazoans to have gap junctions are the cnidarians, specifically members of the class Hydrozoa. Evidence from conventional transmission electron microscopy, lanthanum staining, and freeze fracture work shows these junctions to be structurally closely similar to those of higher animals (Hand and Gobel, 1972; King and Spencer, 1979). Gap junctions are present in electrically coupled glandular epithelium (Mackie, 1976), simple epithelia (Josephson and Schwab, 1974; Satterlie and Spencer, 1983), myoepithelia (Chain *et al.*, 1981; Satterlie and Spencer, 1983) and between certain (but not all) neurons (Spencer and Satterlie, 1980; Spencer, 1981). Dye coupling has been demonstrated in several of these cases.

The most obvious function for gap junctions in hydrozoans is as a pathway for impulse transmission both between coupled neurons and between the cells in electrically

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excitable epithelia which provide the non-nervous conduction pathways which are such a striking feature of hydrozoans (reviewed by Anderson, 1980). Whether they serve a role in metabolic communication is as much an open question here as in other groups. Morphogenetic regulatory molecules have been identified in *Hydra* but it is still not known if they spread within the epithelia, within nerves, or extracellularly (reviewed by Bennett *et al.*, 1981).

Ever since the earliest days of electrical recording from cnidarians it has been clear that the hydrozoans stand sharply apart from the other cnidarians in their electrophysiological characteristics. Josephson (1974) characterizes the dichotomy as follows: "The anthozoans and scyphozoans examined have what might be termed conventional electrophysiology. Signals recorded with extracellular electrodes from conducting systems and muscles are small, generally well under 1 mv, and critically dependent on electrode placement. This is . . . what one would expect for activity in diffuse fibers in a nerve net or thin muscle sheets." In the hydrozoans, on the other hand, conducting systems produce "large electrical signals, typically 1–10 mv. The size of these potentials and their insensitivity to small changes in electrode position indicate that they are generated by blocks of electrogenic epithelia." The cubomedusae, sometimes treated as a fourth cnidarian class (Werner, 1975; Passano, 1982) exhibit electrophysiological responses of the scyphozoan–anthozoan type (Satterlie, 1979; Satterlie and Spencer, 1979).

How are we to account for the existence within one phylum of groups having such profoundly different electrical profiles? In considering this question, it struck us that while the hydrozoan ultrastructure literature is replete with reports of gap junctions, we could recall no such reports from other cnidarian groups. A survey of the literature and discussions with colleagues bears this out. No one to our knowledge has found gap junctions in any cnidarian outside the hydrozoa. Their absence, with few exceptions (*e.g.*, Anderson and Schwab, 1981) has excited no comment.

To satisfy ourselves that the lack of such reports does not simply reflect the use of differing techniques, we have examined tissues from various scyphozoans and anthozoans using a standard procedure (Singla, 1978) that has revealed gap junctions in many hydrozoans. The scyphozoan tissues examined were taken from the arms and tentacles of *Haliclystus steinegeri, Thaumatoscyphus atlanticus,* and the gonads of *Cyanea capillata* and *Rhopilema verrilli.* Developing embryos and planulae of *Cyanea* were also examined. For anthozoans, tentacles from the sea anemones *Aiptasia pulchella* and *Corynactis californica* were investigated. In none of these tissues were gap junctions observed.

Taking these findings at face value, we can immediately see how the electrophysiological differences between hydrozoans and other groups might arise. In hydrozoans, gap junctions would provide close coupling and ready spread of depolarizations within epithelia, whether as propagative events or as local potentials spreading decrementally from neuroeffector junctions. The simultaneous depolarization of such groups of cells would, as Josephson suggested, generate large extracellular signals. The lack of such spread would account for the "conventional electrophysiology" of other cnidarians.

There is no evidence for electrical coupling between cells in anthozoans or scyphozoans. Intracellular recordings from one scyphozoan nerve net, the motor nerve net of *Cyanea capillata,* indicate that there is no coupling between the neurons. Instead, the synapses appear to be chemical (Anderson, unpub.). It has been suggested that the slow conduction systems (SS 1, SS 2) of corals and sea anemones such as *Calliactis* (reviewed by McFarlane, 1982) are neuroid systems of the hydrozoan type, and Shelton (1975) developed a computer model for the SS 1 based on the assumption of electrical coupling in the ectoderm, but none of the workers in this field would

claim that there is any direct evidence for coupling or even for the involvement of the epithelia as the slow conduction pathways.

The failure to observe gap junctions in these groups could merely mean that the junctions are very small, consisting of isolated connexons, or small groups of them. If this were so, the membranes of adjacent cells should show frequent close contacts. Study of the material does not support such a picture.

Alternatively, junctions other than gap junctions (*e.g.,* septate junctions) might provide for electrical communication between cells. Certainly such a possibility cannot be excluded *a priori,* but the available evidence is most easily explained on the assumptions that gap junctions are absent and that coupling, if it exists, must be very loose.

We come then to the hypothesis, which is also a conclusion from existing data, that among the Cnidaria only the hydrozoans have gap junctions. Many questions inevitably arise. Did the common ancestor of the Cnidaria have gap junctions, which survive only in the one class? If so, what led to their elimination in the other classes? If on the other hand the common ancestor lacked gap junctions and they were a hydrozoan invention, does this establish a hydrozoan as ancestor for all higher metazoa?

The gap junction is firmly established as a pathway for electrical communication and, in many cases, transmission of impulses, but what of its supposed role in metabolic communication? Despite their apparent lack of gap junctions the scyphozoans and the anthozoans are no less well organized histologically than the hydrozoans. Presumably, in the absence of direct pathways between cells, tissue communication could still be achieved by interaction of signaling molecules embedded in the membranes of adjacent cells, or by humoral signaling between the cells composing the epithelia (Loewenstein, 1984). Or, finally, tissue regulation could be achieved indirectly by trophic influences from nerves, as in the maintenance of vertebrate skeletal muscle (reviewed by Dennis, 1981). Nutrient transport could be largely extracellular, or could involve amoebocytes. These cells are present in the anthozoa and scyphozoa but are absent in hydrozoans, though in the latter interstitial cells are believed to assume some of the same functions (Chapman, 1974).

Any useful hypothesis should suggest experiments by which it can be tested. The obvious need highlighted by the arguments presented here is for verification of the two fundamental propositions, namely that gap junctions are truly absent from the tissues of scyphozoans and anthozoans and that their cells consequently have little if any capability for *direct* electrical or metabolic communication. If these propositions prove to be true, we will be in a much better position to explain the electrophysiological dichotomy that exists in the phylum, and to plan experiments which might elucidate the mechanisms of metabolic communication within the Cnidaria.

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LITERATURE CITED

ANDERSON, P. A. V. 1980. Epithelial conduction: its properties and functions. *Prog. Neurobiol.* **15:** 161–203.

ANDERSON, P. A. V., AND W. E. SCHWAB. 1981. The organization and structure of nerve and muscle in the jellyfish *Cyanea capillata* (Coelenterata:Scyphozoa). *J. Morphol.* **170:** 383–399.

BENNETT, M. V. L., D. C. SPRAY, AND A. L. HARRIS. 1981. Gap junctions and development. *Trends in Neurosci.* **4:** 159–163.

CHAIN, B. M., Q. BONE, AND P. A. V. ANDERSON. 1981. Electrophysiology of a myoid epithelium in *Chelophyes* (Coelenterata: Siphonophora) *J. Comp. Physiol.* **143:** 329–328.

CHAPMAN, D. M. 1974. Cnidarian histology. Pp. 1–92 in *Coelenterate Biology*, L. Muscatine and H. M. Lenhoff, eds. Academic Press, New York.

DENNIS, M. J. 1981. Development of the neuromuscular junction. Inductive interactions between cells. *Ann. Rev. Neurosci.* **4:** 43–68.

HAND, A. R., AND S. GOBEL. 1972. The structural organization of the septate and gap junctions of *Hydra*. *J. Cell. Biol.* **52:** 397–408.

HYMAN, L. H. 1940. *The Invertebrates: Protozoa through Ctenophora*. McGraw Hill, New York. 726 pp.

JOSEPHSON, R. K. 1974. Cnidarian neurobiology. Pp. 245–280 in *Coelenterate Biology*, L. Muscatine and H. M. Lenhoff, eds. Academic Press, New York.

JOSEPHSON, R. K., AND W. E. SCHWAB. 1979. Electrical properties of an excitable epithelium. *J. Gen. Physiol.* **74:** 213–236.

KING, M. G., AND A. N. SPENCER. 1979. Gap and septate junctions in the excitable endoderm of *Polyorchis penicillatus* (Hydrozoa, Anthomedusae). *J. Cell Sci.* **36:** 391–400.

LOEWENSTEIN, W. R. 1981. Junctional intercellular communication: the cell-to-cell membrane channel. *Physiol. Rev.* **61:** 829–913.

LOEWENSTEIN, W. R. 1984. Cell individuality and connectivity, an evolutionary compromise. Pp. 77–87 in *Individuality and Determinism*, S. W. Fox, ed. Plenum Publ. Corp., New York.

MACKIE, G. O. 1976. Propagated spikes and secretion in a coelenterate glandular epithelium. *J. Gen. Physiol.* **68:** 313–325.

MACKIE, G. O. 1984. Introduction to the diploblastic level. Pp. 43–46 in *Biology of the Integument*, Vol. 1, J. Bereiter-Hahn, A. G. Matoltsy, and K. S. Richards, eds. Springer Verlag, Heidelberg.

MCFARLANE, I. D. 1982. *Calliactis parasitica*. Pp. 243–265 in *Electrical Conduction and Behaviour in 'Simple' Invertebrates*, G. A. B. Shelton, ed. Clarendon Press, Oxford.

PASSANO, C. M. 1982. Scyphozoa and Cubozoa. Pp. 149–202 in *Electrical Conduction and Behaviour in 'Simple' Invertebrates*, G. A. B. Shelton, ed. Clarendon Press, Oxford.

SATTERLIE, R. A. 1979. Central control of swimming in the cubomedusan jellyfish *Carybdea rastonii*. *J. Comp. Physiol.* **133:** 357–367.

SATTERLIE, R. A., AND A. N. SPENCER. 1979. Swimming control in a cubomedusan jellyfish. *Nature* **281:** 141–142.

SCHWARZMANN, G., H. WIEGAND, B. ROSE, A. ZIMMERMAN, D. BEN-HAIM, AND W. R. LOEWENSTEIN. 1981. Diameter of the cell-to-cell junctional membrane channels as probed with neutral molecules. *Science* **213:** 551–553.

SHELTON, G. A. B. 1975. The transmission of impulses in the ectodermal slow conduction system of the sea anemone *Calliactis parasitica* (Couch). *J. Exp. Biol.* **62:** 421–432.

SINGLA, C. L. 1978. Locomotion and neuromuscular system of *Aglantha digitale*. *Cell Tissue Res.* **188:** 317–327.

SPENCER, A. N. 1981. The parameters and properties of a group of electrically coupled neurons in the central nervous system of a hydrozoan jellyfish. *J. Exp. Biol.* **93:** 33–50.

SPENCER, A. N., AND R. A. SATTERLIE. 1980. Electrical and dye coupling in an identified group of neurons in a coelenterate. *J. Neurobiol.* **11:** 13–19.

UNWIN, P. N. T., AND G. ZAMPIGHI. 1980. Structure of the junction between communicating cells. *Nature* **283:** 545–549.

WERNER, B. 1975. Bau and Lebengeschichte des Polypen von *Tripedalia cystophora* (Cubozoa, class. nov., Carybdeidae) und seine Bedeutung für die Evolution der Cnidaria. *Helgol. Wiss. Meeresunters.* **27:** 461–504.

Reference: *Biol Bull.* **167:** 124–138. (August, 1984)

IONIC CONTROL OF SETTLEMENT AND METAMORPHOSIS IN LARVAL *HALIOTIS RUFESCENS* (GASTROPODA)

ANDREA J. BALOUN AND DANIEL E. MORSE

Department of Biological Sciences and the Marine Science Institute, University of California, Santa Barbara, California 93106

ABSTRACT

An increase in the concentration of K^+ in defined sea water medium is demonstrated to induce settlement and metamorphosis in larvae of the marine gastropod mollusc, *Haliotis rufescens.* A decrease in external K^+ ion concentration can inhibit the larval response to γ-aminobutyric acid (GABA), a stereochemically specific inducer of metamorphosis of *H. rufescens.* Stimulation of the metamorphic response by GABA or by increased K^+ may depend on transmembrane movement of ions, since induction is sensitive to neuropharmacological blockers of ion conductance. Sulfonyl isothiocyanostilbene (SITS, an anion exchange blocker) inhibits the larval response to GABA, but does not affect induction by increased external potassium. In contrast, the larval response to potassium is inhibited by tetraethylammonium (TEA, a potassium channel blocker), while induction of metamorphosis by GABA is independent of the presence of TEA. Most manipulations of the concentrations of the other predominant cation components of sea water are not in themselves inductive or inhibitory. However, the actions of GABA and increased K^+ as inducers are sensitive to changes in external Ca^{2+}. Potassium may act by directly depolarizing excitable cells involved in the larval perception of inductive stimuli. Activation of metamorphosis by GABA may depend similarly on a depolarizing ion movement at GABA-sensitive cells. Depolarization by manipulation of the ionic environment may offer a general technique for inducing metamorphosis in various marine invertebrate larvae.

INTRODUCTION

Larval metamorphosis, an essential process in the development of most marine molluscs, is a cascade of complex changes initiated in many cases by specific environmental stimuli (Crisp, 1974; Chia and Rice, 1978). The induction of metamorphosis in larvae of the red abalone, *Haliotis rufescens,* normally depends on the larval encounter of crustose red algae (Morse *et al.,* 1979; 1980c; Morse and Morse, 1984). This inductive action can be mimicked effectively by micromolar concentrations of γ-aminobutyric acid (GABA). When reared at 15°C the planktonic abalone larvae become competent by seven days post-fertilization to respond to the intact alga, algal homogenate, or to micromolar GABA with rapid metamorphosis (Morse *et al.,* 1979, 1980a, b, c). In the continuous presence of an inducer, the larvae cease swimming and attach by the foot to the substrate; this distinct behavioral transition is followed by the characteristic metamorphic sequence described previously (Morse *et al.,* 1980a).

Marine larvae can sense inductive stimuli in the environment, and respond with a coordinated set of behavioral, anatomical, and physiological changes, in a complex process that is likely to involve the larval nervous system (Bonar, 1976; Hadfield, 1978; Burke, 1983a, b). With *Haliotis rufescens,* the direct electrophysiological analysis

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of nervous system involvement is handicapped by the small size of the larvae; we have investigated the function of excitable cells instead by manipulation of ion concentrations and the use of neuropharmacological probes. Evidence presented here demonstrates that the induction of metamorphosis in *H. rufescens* is directly affected by changes in the external concentration of potassium, a physiologically important ion capable of driving both hyperpolarizing and depolarizing shifts in cell membrane potential. The pattern of dose-dependent mimicry or inhibition of GABA action by K^+ is predictable by analogy with the observed influence of K^+ on membrane potential in other excitable cell systems. The sensitivity of induction by GABA to changes in external ion concentration, and to specific neuropharmacological probes, suggests that GABA acts similarly as an excitatory agent, producing depolarization of cells capable of activating metamorphosis. Results obtained with neuropharmacological probes suggest that transmembrane movement of specific ions is required for the activation of metamorphosis by increased K^+ or by GABA. These results are consistent with the hypothesis that the depolarization of externally accessible excitable cells alone is sufficient to initiate behavioral and developmental metamorphosis.

Materials and Methods

Larval culture

Fertilization was controlled by the mixing of washed gametes, spawned by female and male gravid adult *Haliotis rufescens* after a brief exposure to dilute hydrogen peroxide (Morse *et al.*, 1977). Clean healthy cultures of the veliger larvae, maintained in flowing 5 μm-filtered ultraviolet-irradiated sea water at 15.0 ± 1.0°C, synchronously developed to a stage of competence to respond to inducers of metamorphosis by seven days post-fertilization (Morse *et al.*, 1980a).

Artificial sea water media

All experiments were conducted in defined sea water media based on the Woods Hole Marine Biological Laboratory (MBL) recipe (Cavanaugh, 1956). Salt and ion concentrations of this medium are summarized for reference in Table I. Ion concentrations were manipulated by modification of the MBL formula in two ways: (a) ion excess, in which addition of a salt to MBL sea water increased concentrations of the selected anionic and cationic species without reducing the concentrations of MBL sea water components; and (b) ion replacement, in which a single ion species was partially or completely replaced with a molar equivalent of ionic charge by another species (without compensation for differences in dissociation constants). Artificial sea water media were made with reagent grade salts volumetrically diluted in glass-distilled and microfiltered (Barnstead Nanopure) water. The final pH values of all normal and modified MBL media ranged from 7.8 to 8.1 without adjustment. Just prior to use, media were innoculated with the antibiotics potassium penicillin G and dihydrostreptomycin sulfate at 150 ppm each, and equilibrated to 15 ± 1°C.

Assays of induction

All assays were begun with competent veliger larvae (0.2 mm maximum diameter) at 8–10 days post-fertilization. Approximately 200 to 300 larvae were pipetted in a drop of sea water into each 10-ml aliquot of experimental medium, contained in a glass vial (2.4 cm diameter, American Scientific Products). Larvae were incubated in duplicate samples, at 15.0±1.0°C. Induction of plantigrade attachment, assayed as

TABLE I

The salt composition of MBL sea water medium, taken from Cavanaugh (1956) (A), and the calculated maximum free ion concentrations (B)

Component	Concentration (mM)
A. *Salt*	
NaCl	423.0
KCl	9.00
$CaCl_2$	9.27
$MgCl_2$	22.94
$MgSO_4$	25.50
$NaHCO_3$	2.15
B. *Ion*	
Na^+	425.2
K^+	9.00
Ca^{2+}	9.27
Mg^{2+}	48.44
Cl^-	496.4
SO_4^{2-}	25.50

the percentage of larvae firmly attached by the foot, provided a quantitative measure of the larval metamorphic response as a function of time. Completion of metamorphosis was verified by the abcission of the velum (the larval swimming organ) and the initiation of adult shell growth.

Modified sea waters found to produce toxic effects were disqualified from further analysis. Moderate toxicity was recognized in non-induced or pre-metamorphic larvae by absence of the normal swimming behavior: many larvae remained withdrawn in their shells; ciliary activity was decreased; the few swimming larvae moved feebly through the lower water column or spun slowly in circles against the bottom. Larvae introduced into highly toxic conditions remained withdrawn; the rapid paralysis of ciliary and muscular activity was followed by death.

Neuropharmacological agents tested in conjunction with modified sea water media were added to vials and agitated (Vortex mixer) before temperature equilibration and addition of larvae. γ-Aminobutyric acid (GABA), from Sigma Chemical Company, was used at 4×10^{-7} M, a threshold concentration with which facilitation and inhibition are readily detected. SITS (4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonate) was obtained from ICN Nutritional Biochemicals, and tetraethylammonium chloride (TEA) from Eastman Kodak Company. Mallinckrodt Chemical Works analytical reagent grade salts were used in the construction of artificial sea water media, with the exception of the highly hygroscopic salt $MgCl_2 \cdot 6H_2O$ which was purchased as a 4.9 M stock solution from Sigma Chemical Company.

RESULTS

The concentration- and time-dependent responses of larvae to GABA in MBL sea water (Fig. 1) are comparable to the responses of larvae in natural sea water, as defined previously (Morse *et al.*, 1979; 1980a). Typically, 40–60% of the larvae display an attachment response to 4×10^{-7} M GABA by 40 h. Although this value ranges between extremes of 30–90% for different cultures, larval responses within a healthy culture are consistent; variation between duplicate vials remains small.

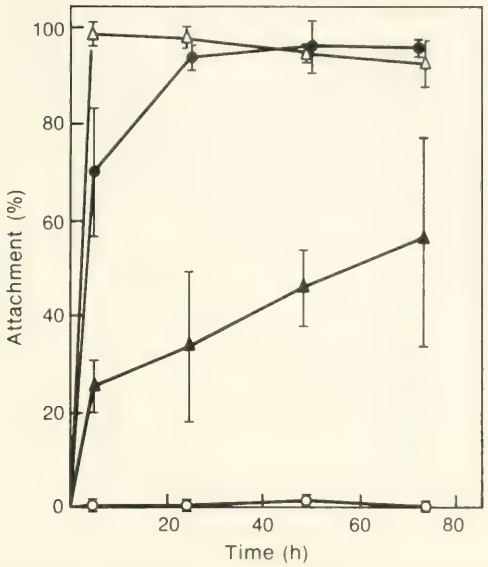

FIGURE 1. Larval attachment in response to GABA in MBL sea water. GABA was added at 10^{-3} M (△), 10^{-6} M (●), 4×10^{-7} M (▲), and 0 M (○). Data are averages of duplicates, with standard deviations indicated by vertical bars.

Ion excess effects

Increased external potassium effectively induced larval attachment, whether added as sulfate or chloride salts to MBL sea water, or used as a replacement for either Na^+ or Mg^{2+} (Fig. 2). In the paired response curves, K^+ added with Cl^- was slightly more efficient as an inducer than when added with SO_4^{2-}. Similarly, the limiting concentration of 20 mM KCl was more rapidly toxic than 10 mM K_2SO_4 (data not included). Both the inductive and toxic effects of K^+ were slightly reduced in medium with sulfate present as the paired anion, instead of chloride.

Increases in the external concentrations of other sea water cations, added in excess to MBL sea water as Cl^- and SO_4^{2-} salts, were not inductive (Table II). With the exception of increased Ca^{2+}, the presence of the various excess salts did not inhibit larval attachment in response to 4×10^{-7} M GABA, indicating that an increase in osmotic pressure alone neither induces nor inhibits induction of metamorphosis. The inhibitory effect of increased Ca^{2+} on induction by GABA corroborates the results obtained from media in which Ca^{2+} concentration was increased by the replacement of Mg^{2+} (as reported below); these results single out Ca^{2+}, rather than concomitant alterations in the substitute or paired salt ion concentration, as the cause of the inhibitory effect.

We have observed that the induction of metamorphosis by excess K^+ is comparable in several respects to that observed with GABA. The efficiency of induction is a dose-dependent function, limited at high concentrations by toxicity. The process of induction involves a temporal component; an optimal concentration of the stimulus (either GABA or increased K^+) must be provided continuously for at least 20 h in order for complete metamorphosis to occur. Premature withdrawal or application of subthreshold levels of the stimulus either fails to induce, or results in only a temporary attachment

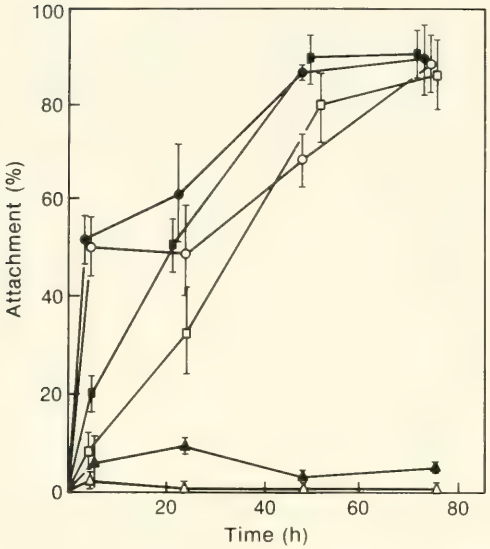

FIGURE 2. Induction of larval attachment by increased external potassium. Potassium was added in excess to MBL sea water as KCl or K_2SO_4 or was used as a replacement for Mg^{2+} or Na^+ in modified MBL sea waters. Excess K^+ was added to MBL sea water as: 2.0 m*M* K_2SO_4 (△), 4.0 m*M* KCl (▲), 6.0 m*M* K_2SO_4 (○), and 12.0 m*M* KCl (●). The effects of increased K^+ concentrations resulting from replacement were tested with media in which: 5.0 mM Mg^{2+} was replaced with 10.0 mM K^+ (□), and 9.0 mM Na^+ was replaced with 9.0 mM K^+ (■). Data are averages of duplicates, with standard deviations indicated as vertical bars.

response that is not followed by completion of metamorphosis. Larvae in media with optimal concentrations of increased K^+ or GABA remain active and responsive; premetamorphic larvae swim normally, while attached larvae in the process of metamorphosis crawl actively on the glass substrate, shed velar lobes, and proceed with growth of new adult shell.

Ion replacement effects

Most cations tested as potential substitutes for sea water cations had toxic effects on larvae and were not used. These ions, replacing either Na^+ or K^+ at concentrations of ≤9 m*M*, included Cs^+, Li^+, choline$^+$, Tris$^+$ [tris(hydroxymethyl)amino-methane], and TEA (tetraethylammonium). However, partial replacements of cations with other MBL sea water cations were tolerated well by the larvae, and were used in tests representing a matrix of exchanges (Table III). Data from two consecutive experiments, which used larvae from two separate hatches, were compiled by normalizing the responses to those obtained with GABA (4×10^{-7} *M*) in unaltered MBL sea water. Each of the four cations present in MBL sea water (Na^+, K^+, Ca^{2+}, Mg^{2+}) was singly replaced by each of the three other species, and the effect of each replacement assayed as a function of time in the presence and absence of 4×10^{-7} *M* GABA. Response groups presented in Table III show consistent patterns of the effects of the cation exchanges: (Group A) normal induction of metamorphosis by 4×10^{-7} *M* GABA in unaltered MBL sea water; (Group B) rapid induction of attachment, with or without GABA present, by increased external potassium (except when replacing calcium); (Group C) inhibition of the attachment response to GABA by reduced external po-

TABLE II

Larval attachment responses in MBL sea water media modified by the addition of excess salts (other than potassium)

Ion excess		Larval attachment (% ± S.D.)	
Salt	Conc. (m*M*)	−GABA	+GABA[1]
None	(MBL sea water)	0 ± 0	72 ± 5
NaCl	10	0 ± 0	52 ± 10
	20	1 ± 0	60 ± 6
	40	1 ± 1	65 ± 6
Na_2SO_4	10	1 ± 1	54 ± 1
	20	2 ± 1	66 ± 10
	40	8 ± 3	64 ± 3
[2]$CaSO_4$	10	2 ± 0	45 ± 21
	20	2 ± 1	35 ± 3
[2]$CaCl_2$	10	0 ± 0	69 ± 11
	20	1 ± 1	28 ± 7
$MgCl_2$	10	0 ± 0	69 ± 13
	20	0 ± 0	66 ± 8
	40	0 ± 0	40 ± 13

[1] Attachment responses are shown at 47 h exposure; GABA was used at 4×10^{-7} *M*.
[2] Calcium salts at 40 m*M* were toxic (as defined in text); larval attachment in these media +GABA was ≤1%.

tassium (except when replaced by magnesium); (Group D) induction, without GABA present, by medium in which sodium was used to replace magnesium, and conversely, inhibition of GABA-induced attachment by medium in which magnesium was replaced by sodium; (Group E) inhibition of the attachment response to GABA by increased external calcium; (Group F) absence of inductive, facilitative, or inhibitory effects of media, without GABA present, in which external calcium concentration was reduced. The absence of inductive ability of increased external K^+ when replacing Ca^{2+} is unique to that exchange condition.

In contrast, decreased potassium (Table III, Group C) was not inductive in any exchange condition. When K^+ was partially replaced by 5.0 m*M* Na^+ or 2.5 m*M* Ca^{2+}, inhibition of GABA action was observed, although increased Ca^{2+} itself also produced inhibition (Group E). The result with substitution by Na^+, however, indicates that decreased external K^+ can inhibit the larval response to GABA.

The only cation replacement capable of inducing attachment of larvae without GABA present, other than replacements resulting in an increase of external potassium, was that in which external Mg^{2+} was replaced with Na^+. The substitution of 23.0 m*M* Mg^{2+} with 46.0 m*M* Na^+ (which permitted the concentration of the paired anion to remain unchanged) was inductive at a level comparable to that of 4×10^{-7} M GABA (Table III, Group C). A comparison with the other substitution conditions in Table III in which Mg^{2+} was decreased, or in which Na^+ was increased, indicates that neither ion shift alone can be credited with the inductive action. Apparently, it is the specific replacement of Mg^{2+} with Na^+ that effects larval attachment. The reverse replacement of 46.0 m*M* Na^+ with 23.0 m*M* Mg^{2+} strongly inhibited induction by 4×10^{-7} *M* GABA. Again, a comparison of the results obtained with other media in which external Na^+ was decreased, or Mg^{2+} was increased, shows that neither cation change alone is consistently inhibitory.

TABLE III

Larval attachment responses in MBL sea water media modified by cation replacement

Replacement media					Larval response[1]			
					−GABA		+GABA	
Group	Cation replaced	Conc. (m*M*)	Cation substituted	Conc. (m*M*)	Relative attachment (% ± S.D.)[2]	Effect[3]	Relative attachment (% ± S.D.)[2]	Effect[3]
A	(MBL sea water control; no replacement)				0 ± 0.0	N	100 ± 8.4	N
B	Na^+	9	K^+	9	143 ± 5.6	I	148 ± 2.8	F
	Na^+	18	K^+	18	152 ± 7.0	I	143 ± 7.0	F
	Mg^{2+}	5	K^+	10	159 ± 4.2	I	158 ± 1.4	F
	Ca^{2+}	5	K^+	10	0 ± 0.0	N	101 ± 1.4	N
C	K^+	5	Na^+	5	0 ± 0.0	N	61 ± 13	X
	K^+	5	Mg^{2+}	2.5	0 ± 0.0	N	92 ± 21	N
	K^+	5	Ca^{2+}	2.5	0 ± 0.0	N	59 ± 4.2	X
D	Mg^{2+}	23	Na^+	46	97 ± 9.8	I	117 ± 1.4	N
	Na^+	46	Mg^{2+}	23	0 ± 0.0	N	10 ± 2.8	X
E	Mg^{2+}	23	Ca^{2+}	23	4 ± 1.4	N	62 ± 2.8	X
	Na^+	23	Ca^{2+}	11.5	0 ± 0.0	N	46 ± 7.0	X
	[4]K^+	5	Ca^{2+}	2.5	0 ± 0.0	N	59 ± 4.2	X
F	Ca^{2+}	5	Na^+	10	0 ± 0.0	N	92 ± 11	N
	Ca^{2+}	5	Mg^{2+}	5	0 ± 0.0	N	89 ± 1.4	N
	[4]Ca^{2+}	5	K^+	10	0 ± 0.0	N	101 ± 1.4	N

[1] Attachment responses are shown at 45 h exposure; GABA was used at 4×10^{-7} *M*.

[2] Normalized to attachment observed in MBL sea water + GABA, as explained in Results; the absolute value of attachment in MBL seawater + GABA was 47%. S.D. is the absolute standard deviation.

[3] Effects: (N) no facilitating or inhibitory effect compared with response in unmodified MBL sea water; (I) induction without GABA; (F) facilitation of induction by GABA; (X) inhibition of induction by GABA.

[4] Media listed twice for comparison in separate groups.

TABLE IV

The effects of altered concentrations of external Ca^{2+} on larval attachment responses to GABA and to increased K^+

Altered ion concentrations[1]				Larval attachment (% ± S.D.)					
				−GABA			+GABA[2]		
K^+	Na^+	Ca^{2+}	Mg^{2+}	22 h	49 h	72 h	22 h	49 h	72 h
None (MBL sea water)				0 ± 0	1 ± 1	0 ± 0	60 ± 13	80 ± 21	89 ± 4
		+4	−4	0 ± 0	0 ± 0	0 ± 0	36 ± 2	88 ± 6	94 ± 0
		+9	−9	0 ± 0	1 ± 1	0 ± 0	11 ± 6	41 ± 3	52 ± 13
+12	−12			62 ± 12	57 ± 3	74 ± 1	79 ± 3	90 ± 3	94 ± 1
+12	−12	+4	−4	63 ± 3	80 ± 4	93 ± 3	75 ± 0	95 ± 1	96 ± 3
+12	−12	+9	−9	57 ± 12	81 ± 1	96 ± 3	64 ± 16	83 ± 9	95 ± 4
		−4	+4	0 ± 0	1 ± 1	0 ± 0	41 ± 17	69 ± 20	95 ± 2
		−9	+9	0 ± 0	0 ± 0	0 ± 0	0 ± 0	1 ± 0	0 ± 0
+12	−12	−4	+4	5 ± 2	4 ± 3	2 ± 2	72 ± 2	82 ± 0	87 ± 2
+12	−12	−9	+9	2 ± 1	0 ± 0	0 ± 0	9 ± 1	43 ± 13	44 ± 10

[1] Changes in cation concentration (m*M*) with reference to standard MBL sea water.

[2] GABA was used at 4×10^{-7} *M*.

The actions of GABA and increased external K^+ as inducers of metamorphosis both were inhibited by changes in external Ca^{2+}, although the directions of the net change in Ca^{2+} to which they were sensitive were opposite (Table IV). Without an inducer present, the changes in external Ca^{2+} (imposed in combination with reciprocal equimolar changes in external Mg^{2+}) had no effect on exposed larvae. Larval attachment in response to 4×10^{-7} *M* GABA was inhibited by a 9.0 m*M* increase in Ca^{2+}. Larval responses to increased external K^+ (introduced as an equimolar replacement for Na^+, without GABA present) were not affected by increased external Ca^{2+}, indicating that inhibition of the response to GABA was not caused by toxicity. In contrast, the larval response to GABA in medium with Ca^{2+} decreased by 4.0 m*M* remained comparable to that in MBL sea water with GABA. However, the larval response to increased K^+ was strongly inhibited by the 4.0 m*M* reduction in Ca^{2+}. Despite this strong inhibition, the normal response of larvae to GABA (present in addition to the increased K^+ and decreased Ca^{2+}) was retained, again negating the possibility that toxicity was the cause of inhibition. Virtually complete replacement of Ca^{2+} (−9.0 m*M*) inhibited attachment in all conditions, suggesting that this extreme reduction in external Ca^{2+} was detrimental to the larvae.

Neuropharmacological analyses

Neuropharmacological probes were used to analyze the effects of external ion changes in the initiation of metamorphosis. Induction by GABA is sensitive specifically to the presence of SITS, an isothiocyanate derivative known to inhibit anion exchange (Cabantchik and Rothstein, 1972). Addition of 1×10^{-5} *M* SITS to MBL sea water inhibited larval attachment in response to GABA, without altering larval behavior in the absence of GABA (Table V). In contrast, the induction of metamorphosis by increased potassium was not affected by SITS. The presence of SITS did not substantially reduce the increase in larval attachment contributed by GABA, when present in addition to 12 m*M* excess K^+.

The effectiveness of SITS as an inhibitor of GABA action depends on its concentration relative to that of GABA (Fig. 3). SITS at 10^{-4} *M* fully blocked the inductive effect of 10^{-4} *M* GABA. A concentration of SITS lower by one order of magnitude (10^{-5} *M*) did not block induction by 10^{-4} *M* GABA but did affect the rate of attachment induced by lower concentrations of GABA. SITS at 10^{-6} *M* was relatively ineffective,

TABLE V

The effects of a K^+-channel blocker (TEA) and an anion exchange blocker (SITS) on larval attachment responses to increased K^+ and GABA

KCl Excess[1]	GABA	Larval attachment (% ± S.D.)[2]		
		Alone	+TEA	+SITS
0	0	0 ± 0	0 ± 0	0 ± 0
	4×10^{-7} *M*	43 ± 10	47 ± 18	16 ± 1
12 m*M*	0	54 ± 10	24 ± 11	57 ± 7
	4×10^{-7} *M*	82 ± 6	43 ± 3	75 ± 0

[1] MBL sea water media prepared as described in text.

[2] Absolute percentage of larvae attached after 24 h exposure; S.D. is standard deviation. Concentrations of additions: TEA (5×10^{-5} *M*); SITS (1×10^{-5} *M*).

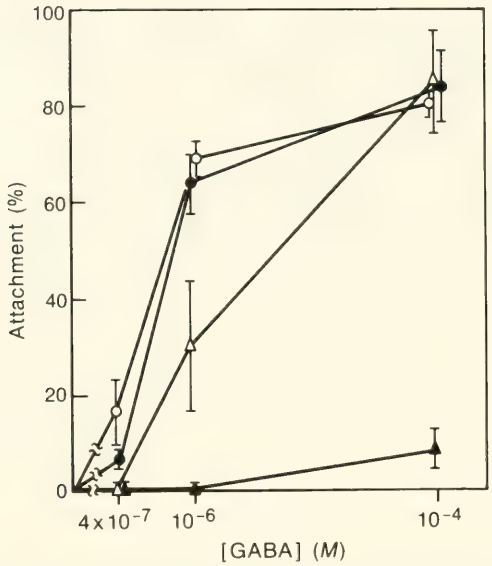

FIGURE 3. Inhibition by SITS, as a function of the relative concentrations of SITS and GABA. GABA concentrations are indicated on the horizontal axis. Larval attachment at 28 h in response to GABA is shown for media in which SITS is present at concentrations of: 10^{-4} *M* (▲), 10^{-5} *M* (△), 10^{-6} *M* (●), and 0 *M* (○). Data are averages of duplicates, with standard deviations indicated as vertical bars.

except when present with the threshold concentration of 4×10^{-7} *M* GABA. SITS at concentrations lower than those of GABA seemed to have little inhibitory influence.

At the erythrocyte membrane, the covalent binding of isothiocyanate groups to the anion transporter protein occurs at specific amino groups (Passow *et al.*, 1982). The possibility that SITS might inhibit the larval response to GABA by binding the γ-amino group of GABA and thus decreasing the effective concentration, rather than by acting at larval membrane sites, was tested using glycine, a non-inductive and non-facilitating structural analog. Glycine, added with SITS for a one hour preincubation prior to the addition of GABA and competent larvae, remained continuously present during the subsequent assays of induction. No competitive protection of induction by GABA from inhibition by SITS was evident in the presence of glycine; SITS fully retained its ability to inhibit GABA action (Fig. 4). Glycine alone had no effect on induction by GABA. The possibility that SITS might act to bind GABA, but not glycine, because of steric hindrance of the amino group in the shorter molecule, was tested by repeating the protocol with ε-aminocaproic acid (a longer homolog of GABA) instead of glycine; the identical result further shows that SITS does not act by binding nonspecifically to the amino groups of amino acids in solution. The inhibitory action of SITS appears to be relatively specific. Other potential blockers of ion conductance that were found to have no effect on the normal larval response to GABA include: (a) tetrodotoxin, a blocker of voltage-regulated sodium channels in axonal membranes (review by Armstrong, 1974); (b) picrotoxin, a blocker of GABA-regulated increases in Cl^- permeability in some systems (Takeuchi, 1976; Gallagher *et al.*, 1978; Yarowsky and Carpenter, 1978); and (c) furosemide, an inhibitor of mediated cotransport (Geck *et al.*, 1980).

The action of potassium in the induction of metamorphosis was analyzed using

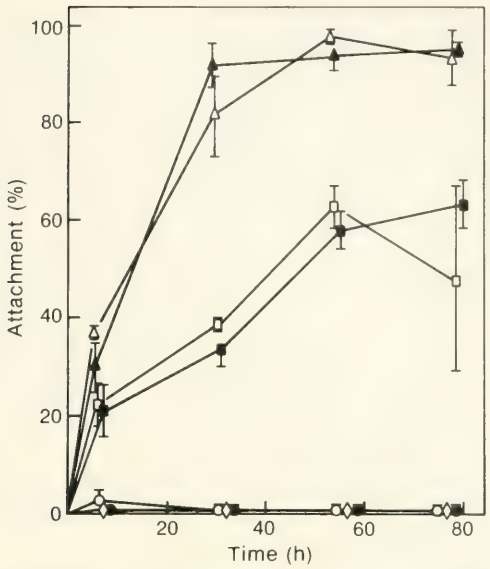

FIGURE 4. The inhibitory action of SITS on the larval response to GABA, with or without preincubation of SITS with glycine. SITS and/or glycine, where indicated, were added 1 h before initiation of the experimental assay by addition of GABA, where indicated, and subsequent introduction of competent larvae. Larval responses are shown for MBL sea water with: no addition (○); glycine (●); GABA (△); GABA and glycine (▲); SITS (◇); SITS and GABA (□); SITS and GABA with glycine (■). Concentrations were: GABA 4×10^{-7} *M;* glycine, 10^{-5} M; and SITS, 10^{-5} *M*. Data are averages of duplicates, with standard deviations indicated as vertical bars.

tetraethylammonium chloride (TEA), an impermeant blocker of K^+ channels in nerve and muscle cells (review by Armstrong, 1974); both intracellular and extracellular applications of TEA block the Ca^{2+}-activated K^+ current in molluscan neurons (Hermann and Gorman, 1981). At concentrations less than 10^{-4} *M,* TEA specifically inhibits induction of *H. rufescens* by increased K^+; higher concentrations of TEA are toxic, and cause non-specific inhibition of larval responses to all inducers. Concentrations of TEA less than 10^{-5} *M* have no apparent inhibitory effect. The presence of 5×10^{-5} *M* TEA reduced the inductive action of 12 m*M* excess KCl and negated the additive effect of increased K^+ when present in combination with 4×10^{-7} *M* GABA, reducing the attachment to a level equivalent to that of GABA in MBL sea water alone (Table V). Induction by GABA in MBL sea water was unaffected by the presence of TEA at 5×10^{-5}, indicating that inhibition by TEA does not result from toxicity. Function of the TEA-sensitive sites thus is required for the induction of metamorphosis by increased K^+, but apparently is not essential for the pathway activated by GABA.

DISCUSSION

The complete process of metamorphosis is induced in *Haliotis rufescens* larvae by an increase in the concentration of K^+ in sea water. Changes in external K^+ concentration can drive electrogenic movements of K^+ that directly affect the membrane potential; the depolarization of membrane potential as a function of increasing extracellular K^+ has been used to demonstrate that the excitable membrane can behave in a classical sense as a K^+ electrode (Hodgkin and Horowicz, 1959). The

inductive action of increased K^+ suggests that metamorphosis in *H. rufescens* can be initiated solely by the depolarization of externally accessible excitable cells. Depolarizing electrical stimuli, delivered by suction electrode to the region of the oral ganglion or apical neuropile, have been shown by Burke (1983a) to elicit immediate metamorphosis in competent larvae of the Pacific sand dollar *Dendraster excentricus*. This site-specific efficacy suggests that the metamorphic response to an appropriate environmental stimulus is activated in this species by the neural communication of sensory receptors with the larval nervous system.

The induction of metamorphosis of *H. rufescens* by GABA may depend similarly on the depolarization of GABA-sensitive cells. The initial larval response to GABA in the presence of increased external K^+ is greater than that observed with either GABA or increased K^+ alone (Table V). In contrast to this combined effect of increased K^+ with GABA, a decrease in external K^+ can inhibit induction by GABA. Hyperpolarization resulting from the decrease in external potassium, as demonstrated for GABA-regulated postsynaptic cells (Motokizawa *et al.*, 1969), could antagonize a GABA-mediated depolarization. It is unlikely that GABA acts directly by altering membrane permeability to K^+ at the same sites utilized during induction by increased K^+, since the actions of these inducers are pharmacologically separable. Induction by GABA is sensitive to SITS, and insensitive to TEA; induction by increased K^+ is inhibited by TEA but not by SITS. These reciprocal sensitivities also indicate that the inducers operate through pathways that initially are separate; that is, neither follows the other in an obligatory sequence in the process of induction.

The separateness of the inductive actions of GABA and increased K^+ also is evident in their entirely different sensitivities to alterations in external Ca^{2+}. Induction by GABA is inhibited specifically by increased Ca^{2+}, while induction by increased K^+ is sensitive only to a reduced external concentration of Ca^{2+}. A simple model can be proposed, analogous to other systems, which invokes a single mechanism to explain the opposite sensitivities of these inducers. Increased cytoplasmic concentrations of Ca^{2+} have been shown to activate K^+ conductance through Ca^{2+}-regulated K^+ channels in diverse cell types (review by Schwarz and Passow, 1983). At physiological concentrations of internal and external K^+, a Ca^{2+}-activated increase in K^+ conductance permits a net K^+ efflux that can hyperpolarize a sensory receptor cell, thus decreasing the rate of afferent discharge (review by Edwards, 1984). If we postulate the existence, in larval *H. rufescens*, of Ca^{2+}-regulated K^+ channels in cells that are capable of responding to GABA and to K^+, then the effects of Ca^{2+} can be explained by a comparable mechanism. In medium with a standard sea water concentration of K^+, an increase in calcium (suggested to produce a parallel increase in cytoplasmic calcium) may inhibit the effect of GABA by activating a hyperpolarizing net K^+ efflux. With an inductive increase in external K^+, however, membrane depolarization rather than hyperpolarization would be expected in response to increased Ca^{2+}; this prediction is supported by the observed absence of an inhibitory effect of increased Ca^{2+} on induction by K^+. In contrast, a decrease in external Ca^{2+} (suggested to produce a decrease in cytoplasmic Ca^{2+}) may block induction by K^+ by antagonizing the necessary electrogenic influx of the cation through Ca^{2+}-regulated membrane channels. The reduced efficiency of K^+ as an inducer, when added with sulfate rather than chloride to MBL sea water, may result from a decrease in the sea water concentration of free Ca^{2+}, since $CaSO_4$ has a higher association constant than $CaCl_2$. The induction of metamorphosis by GABA is not sensitive to decreased Ca^{2+}, suggesting that its action is not impaired by an increased membrane resistance to K^+; this idea is supported by our demonstration that induction by GABA is insensitive to the presence of the K^+-channel blocker, TEA. Although a heterogeneous population of larval cells is exposed during the test of an altered sea water medium, the resulting

effects on larval metamorphosis are consistent with this model based on the exclusive function of a single group of accessible excitable cells.

The selective movement of ions across specialized membranes is a fundamental mechanism in the function of excitable cells. At invertebrate chemoreceptors, a stimulus-dependent increase in ion permeability can transduce chemical stimuli into electrical impulses, allowing nervous system analysis of environmental information (Morita, 1972; Thurm and Wessel, 1979; Kaissling and Thorson, 1980). Postsynaptic cells mediate the effect of a chemical neurotransmitter similarly by altering membrane permeability to ions capable of influencing the membrane potential (Takeuchi and Takeuchi, 1960). GABA, as an inhibitory neurotransmitter in both vertebrate and invertebrate systems, acts at postsynaptic sites to increase membrane permeability to chloride (Krnjević and Schwartz, 1967; Takeuchi *et al.*, 1978; review by Takeuchi, 1976). While the inhibitory effect of GABA commonly depends on a hyperpolarizing Cl^- influx, GABA also has been shown to activate a depolarizing efflux of Cl^- in the presynaptic inhibition of vertebrate spinal ganglia (Nishi *et al.*, 1974; Gallagher *et al.*, 1978). GABA can hyperpolarize or depolarize different cells within the same ganglion in invertebrates such as *Helix* (Walker *et al.*, 1975) and *Cancer* (Marder and Paupardin-Tritsch, 1978).

The site of action of exogenous GABA as an inducer of metamorphosis of *H. rufescens* larvae remains unknown. If acting through the larval nervous system, GABA is likely to function either as a ligand mimicking the active component of the inductive algae at larval chemoreceptors, or as a neurotransmitter at synapses between neurons regulating the initiation of metamorphosis. We have shown that the larval response to GABA depends on the function of a SITS-sensitive process. This requirement appears to be specific, since induction by increased K^+ is not inhibited by SITS, and potential blockers of other ion conductances fail to inhibit the larval response to GABA. It is possible that the larval response to GABA may be directly dependent on a GABA-controlled alteration of SITS-sensitive anion exchange. Although anion exchange processes generally are considered to be electrically neutral, GABA could generate the depolarizing net efflux of an anion such as Cl^- by promoting "slippage," an exchanger-mediated process in which the unidirectional transport of an anion occurs without an associated anion countertransport (Knauf *et al.*, 1977; Fröhlich *et al.*, 1983). Alternatively, the SITS-sensitive system may not be directly controlled by GABA, but may be capable of influencing a state or process on which GABA action does depend.

Other data support the suggestion that there may be a functional relationship between GABA as an inducer of metamorphosis, and the transmembrane movement of anions. We have found that the induction of metamorphosis of *H. rufescens* larvae by GABA is sensitive to changes in Cl^- concentration in artificial sea water. Furthermore, without GABA present, the replacement of 25–75% of Cl^- in sea water with substitute anions (Br^-, SO_4^{2-}, NO_3^-, acetate, isethionate, or propionate) induces attachment of competent larvae; this inductive action is inhibited by SITS, but not by TEA. The macrocyclic lactone ivermectin, a compound demonstrated to increase Cl^- conductance at a GABA-regulated synapse in lobster (Fritz *et al.*, 1979), is inductive alone and facilitates induction by media in which Cl^- is replaced with a substitute anion. An increase in external Cl^-, added in excess with Mg^{2+}, blocks induction by GABA. These results, suggesting that Cl^- efflux may play a role in transduction of the GABA signal, will be presented in more detail elsewhere (Baloun and Morse, in prep.).

The data presented here support the idea that GABA and increased K^+ work similarly by causing depolarization, but require the function of different ion-conductive processes in the induction of metamorphosis. Additional work will be required to

determine how the specific exchanges of Mg^{2+} and Na^+ either induce attachment of larvae or block the response to GABA. The induction of larval attachment by medium in which Mg^{2+} is replaced with Na^+ is insensitive both to SITS and TEA, suggesting a mechanism of action separate from those of GABA and increased K^+.

The culture of marine invertebrates, for research in ontogeny and neurobiology, and for production of food and other resources, would benefit from the development of a general technique for initiating larval metamorphosis. The nature of the specific inducing signals naturally required for metamorphosis is likely to vary among species recruited to different specialized microenvironments. In contrast, the transduction of chemical or other stimuli by receptor cell depolarization may be a more general mechanism in initiating the metamorphic response. The depolarizing effect of small increases in external K^+ thus may provide a simple and economical method for the induction of metamorphosis in a variety of marine invertebrates. This idea is supported by our recent finding that larvae of the gastropod mollusc *Astraea undosa,* for which the natural inducer is not yet identified, efficiently are induced to settle and metamorphose in a dose-dependent response to increased external potassium (Markell, Baloun, and Morse, unpubl. obs.). The optimal concentration of excess potassium required for the metamorphic response of *A. undosa* is close to that described here for *Haliotis.*

While the specific physical and chemical characteristics of substrates influencing settlement of marine invertebrate larvae have been extensively reviewed (Crisp, 1974; Scheltema, 1974; Hadfield, 1978), few studies are available on the role of neurophysiologically important ions in larval induction. Early work by Lynch (1947) suggested that influx of Na^+ during brief exposure to greater than normal concentrations of sodium salts could accelerate metamorphosis of *Bugula* larvae. Spindler and Müller (1972) demonstrated an inductive response to LiCl in planula larvae of *Hydractinia echinata.* Subsequent work by Müller and Buchal (1973) defined a range of inductive responses to Cs^+, Rb^+, Li^+, and K^+. Succinyl choline chloride was shown to induce metamorphosis in *Phestilla* larvae (Bonar, 1976); the active component choline is inductive alone, although less efficient than the natural inducer of metamorphosis (Hadfield, 1978). In these and related studies, the mechanisms of action of the inductive ion changes have remained hypothetical (Müller and Buchal, 1973), or were considered to be unrelated to the normal physiological mechanism (Crisp, 1974, 1984; Hadfield, 1978, 1984). Our results with larvae of *Haliotis rufescens* suggest that these results obtained in other systems, once considered to be artifactual, may in retrospect be recognized as clues to the integral role of ions in transducing the environmental stimuli required for metamorphosis.

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LITERATURE CITED

ARMSTRONG, C. M. 1974. Ionic pores, gates, and gating currents. *Q. Rev. Biophys.* **7**: 179–210.

BONAR, D. B. 1976. Molluscan metamorphosis: A study in tissue transformation. *Am. Zool.* **16**: 573–591.

BURKE, R. D. 1983a. Neural control of metamorphosis in *Dendraster excentricus*. *Biol. Bull.* **164**: 176–188.

BURKE, R. D. 1983b. The induction of marine invertebrate larvae: stimulus and response. *Can. J. Zool.* **61**: 1701–1719.

CABANTCHIK, Z. I., AND A. ROTHSTEIN. 1972. The nature of membrane sites controlling anion permeability of human red blood cells as determined by studies with disulfonic stilbene derivatives. *J. Memb. Biol.* **10**: 311–330.

CAVANAUGH, G. M. 1956. Pp. 62–69 in *Formulae and Methods IV of the Marine Biological Laboratory Chemical Room*. Woods Hole, Massachusetts.

CHIA, F. S., AND M. E. RICE, eds. 1978. *Settlement and Metamorphosis of Marine Invertebrate Larvae*. Elsevier, New York.

CRISP, D. J. 1974. Factors influencing settlement of marine invertebrate larvae. Pp. 177–265 in *Chemoreception in Marine Organisms*, P. T. Grant and A. M. Mackie, eds. Academic Press, New York.

CRISP, D. J. 1984. Overview of research on marine invertebrate larvae, 1940–1980. Pp. 103–126 in *Marine Biodeterioration*, J. D. Costlow and R. C. Tipper, eds. Naval Institute Press, Annapolis, Maryland.

EDWARDS, C. 1983. The ionic mechanisms underlaying the receptor potential in mechanoreceptors. Pp. 497–503 in *The Physiology of Excitable Cells*, A. D. Grinnell and W. J. Moody Jr., eds. Alan R. Liss, Inc., New York.

FRITZ, L. C., C. C. WANG, AND A. GORIO. 1979. Avermectin $B_{1}a$ irreversibly blocks postsynaptic potentials at the lobster neuromuscular junction by reducing muscle membrane resistance. *Proc. Natl. Acad. Sci. USA* **76**(4): 2062–2066.

FRÖHLICH, O., C. LEIBSON, AND R. B. GUNN. 1983. Chloride net efflux from intact erythrocytes under slippage conditions: Evidence for a positive charge on the anion binding/transport site. *J. Gen. Physiol.* **81**: 127–152.

GALLAGHER, J. P., H. HIGASHI, AND S. NISHI. 1978. Characterization and ionic basis of GABA-induced depolarizations recorded "in vitro" from cat primary afferent neurones. *J. Physiol.* **275**: 263–282.

GECK, P., C. PIETRTYK, B. C. BURCKHARDT, B. PFEIFFER, AND E. HEINZ. 1980. Electrically silent cotransport of Na^+, K^+ and Cl^- in Ehrlich cells. *Biochim. Biophys. Acta* **600**: 432–447.

HADFIELD, M. G. 1978. Metamorphosis in marine molluscan larvae: an analysis of stimulus and response. Pp. 165–175 in *Settlement and Metamorphosis of Marine Invertebrate Larvae*, F. S. Chia and M. E. Rice, eds. Elsevier, New York.

HADFIELD, M. G. 1984. Settlement requirements of molluscan larvae: New data on chemical and genetic roles. In *Recent Innovations in Cultivation of Pacific Molluscs*, D. Morse, K. Chew, and R. Mann, eds. Elsevier, New York. (In press.)

HERMANN, A., AND A. L. F. GORMAN. 1981. Effects of tetraethylammonium on potassium currents in a molluscan neuron. *J. Gen. Physiol.* **78**: 87–110.

HODGKIN, A. L., AND P. HOROWICZ. 1959. The influence of potassium and chloride ions on the membrane potential of single muscle fibres. *J. Physiol.* **148**: 127–160.

KAISSLING, K. E., AND J. THORSON. 1980. Insect olfactory sensilla: structural, chemical and electrical aspects of the functional organization. Pp. 261–282 in *Receptors for Neurotransmitters, Hormones and Pheromones in Insects*, D. B. Satelle *et al.*, eds. Elsevier/North-Holland Biomedical Press, New York.

KNAUF, P. A., G. F. FUHRMANN, S. ROTHSTEIN, AND A. ROTHSTEIN. (1977). The relationship between anion exchange and net anion flow across the human red blood cell membrane. *J. Gen. Physiol.* **69**: 363–386.

KRNJEVIĆ, K., AND S. SCHWARTZ. 1967. The action of γ-aminobutyric acid on cortical neurones. *Exp. Brain Res.* **3**: 320–336.

LYNCH, W. F. 1947. The behavior and metamorphosis of the larva of *Bugula neritina* (Linnaeus): Experimental modification of the length of the free-swimming period and the responses of the larvae to light and gravity. *Biol. Bull.* **92**: 115–150.

MARDER, E., AND D. PAUPARDIN-TRITSCH. 1978. The pharmacological properties of some crustacean neuronal acetylcholine, γ-aminobutyric acid, and L-glutamate responses. *J. Physiol.* **280**: 213–236.

MORITA, H. 1972. Primary processes of insect chemoreception. *Adv. Biophys.* **3**: 161–198.

MORSE, A., AND D. E. MORSE. 1984. Recruitment and metamorphosis of *Haliotis* larvae are induced by molecules uniquely available at the surfaces of crustose red algae. *J. Exp. Mar. Biol. Ecol.* **75**: 191–215.

MORSE, D. E., H. DUNCAN, N. HOOKER, AND A. MORSE. 1977. Hydrogen peroxide induces spawning in molluscs, with activation of prostaglandin endoperoxide synthetase. *Science* **196:** 298–300.

MORSE, D. E., N. HOOKER, H. DUNCAN, AND L. JENSEN. 1979. γ-Aminobutyric acid, a neurotransmitter, induces planktonic abalone larvae to settle and begin metamorphosis. *Science* **204:** 407–410.

MORSE, D. E., H. DUNCAN, N. HOOKER, A. BALOUN, AND G. YOUNG. 1980a. GABA induces behavioral and developmental metamorphosis in planktonic molluscan larvae. *Fed. Proc.* **39:** 3237–3241.

MORSE, D. E., N. HOOKER, AND H. DUNCAN. 1980b. GABA induces metamorphosis in *Haliotis*, V: Stereochemical specificity. *Brain Res. Bull.* 5, Suppl. **2:** 381–387.

MORSE, D. E., M. TEGNER, H. DUNCAN, N. HOOKER, G. TREVELYAN, AND A. CAMERON. 1980c. Induction of settling and metamorphosis of planktonic molluscan larvae III: Signaling by metabolites of intact algae is dependent on contact. Pp. 67–86 in *Chemical Signaling in Vertebrate and Aquatic Animals*, D. Müller-Schwarze and R. M. Silverstein, eds. Plenum Press, New York.

MOTOKIZAWA, F., J. P. REUBEN, AND H. GRUNDFEST. 1969. Ionic permeability of the inhibitory postsynaptic membrane of lobster muscle fibers. *J. Gen. Physiol.* **54:** 437–461.

MÜLLER, W. A., AND G. BUCHAL. 1973. Metamorphose-induktion bei Planulalarven. II. Induktion durch monovalente Kationen: Die Bedeutung des Gibbs-Donnan-Verhaltnisses und der Na^+/K^+-ATPase. *Wilhelm Roux' Arch.* **174:** 122–135.

NISHI, S., S. MINOTA, AND A. G. KARCZMAR. 1974. Primary afferent neurones: The ionic mechanism of GABA-mediated depolarization. *Neuropharmacology* **13:** 215–219.

PASSOW, H., H. FASOLD, M. L. JENNINGS, AND S. LEPKE. 1982. The study of the anion transport protein ("band 3 protein") in the red blood cell membrane by means of tritiated 4,4′-diisothiocyano-dihydrostilbene-2,2′-disulfonic acid ($^3H_2$-DIDS). Pp. 1–31 in *Chloride Transport in Biological Membranes*, J. A. Zadunaisky, ed. Academic Press, New York.

SCHELTEMA, R. S. 1974. Biological interactions determining larval settlement of marine invertebrates. *Thallasia Jugoslav.* **10:** 263–296.

SCHWARZ, W., AND H. PASSOW. 1983. Ca^{2+}-activated K^+ channels in erythrocytes and excitable cells. *Ann. Rev. Physiol.* **45:** 359–374.

SPINDLER, K-D., AND W. A. MÜLLER. 1972. Induction of metamorphosis by bacteria and by a lithium-pulse in the larvae of *Hydractinia echinata* (Hydrozoa). *Wilhelm Roux' Arch.* **169:** 271–280.

TAKEUCHI, A. 1976. Studies of inhibitory effects of GABA in invertebrate nervous systems. Pp. 255–267 in *GABA in Nervous System Function*, E. Roberts, T. N. Chase, D. B. Tower, eds. Raven Press, New York.

TAKEUCHI, A., AND N. TAKEUCHI. 1960. On the permeability of the end-plate membrane during the action of the transmitter. *J. Physiol. Lond.* **154:** 52–67.

TAKEUCHI, H., K. WATANABE, AND H. TAMURA. 1978. Penetrable and impenetrable anions into the GABA-activated chloride channel on the postsynaptic neuromembrane of an identifiable giant neurone of an African giant snail (*Achatina fulica* Ferussac). *Comp. Biochem. Physiol.* **61C:** 309–315.

THURM, U., AND G. WESSEL. 1979. Metabolism-dependent transepithelial potential differences at epidermal receptors of arthropods. *J. Comp. Physiol.* **134A:** 119–130.

WALKER, R. J., M. J. AZANZA, G. A. KERKUT, AND G. N. WOODRUFF. 1975. The action of γ-aminobutyric acid (GABA) and related compounds on two identifiable neurones in the brain of the snail *Helix aspera*. *Comp. Biochem. Physiol.* **50C:** 147–154.

YAROWSKY, P. J., AND D. O. CARPENTER. 1978. A comparison of similar ionic responses to γ-aminobutyric acid and acetylcholine. *J. Neurophysiol.* **41:** 531–541.

Reference: *Biol Bull.* **167:** 139–158. (August, 1984)

BIOLOGY OF HYDRACTINIID HYDROIDS. 2. HISTOCOMPATIBILITY EFFECTOR SYSTEM/COMPETITIVE MECHANISM MEDIATED BY NEMATOCYST DISCHARGE

LEO W. BUSS, CATHERINE S. MCFADDEN[1], AND DOUGLAS R. KEENE[2]

Department of Biology, Yale University, New Haven, Connecticut 06511

Abstract

Intraspecific encounters between colonies of the athecate, colonial hydroid *Hydractinia echinata* result in contact between mat or stolonal tissues. We have monitored colony ontogeny in five clones of *H. echinata* and initiated experimental encounters between the two tissue types in both isogeneic and allogeneic combinations. All isogeneic interactions result in fusion, all allogeneic interactions in rejection. Transmission electron microscopy shows that fusion results in the establishment of a common gastrovascular system, whereas rejection is characterized by an electron-dense, fibrous layer separating the two colonies. Rejection involves either the passive cessation of growth along the contact zone or the development of hypertrophied stolons. These hyperplastic stolons destroy foreign tissues and can develop only from existing stolons. Scanning and transmission electron microscopy demonstrates that stolons become hyperplastic through the differentiation of interstitial cells into nematocytes and that the destruction of foreign tissue is effected by nematocyst discharge. Experimental elimination of interstitial cells removes the capacity of a colony to produce hyperplastic stolons, but does not affect historecognition. A comparison between these results and similar studies in anthozoans suggests the need to distinguish between the evolution of historecognition and the evolution of mechanisms of interference competition.

Introduction

Cnidarians have evolved a striking array of behavioral repertoires and morphological structures to defend their living space and expand into the space occupied by others. Scleractinian corals contacting other scleractinians extrude mesenterial filaments and actively digest their neighbors (Lang, 1971, 1973; Glynn, 1976; Sheppard, 1979). Scleractinians may also differentiate sweeper tentacles along zones of contact. These modified tentacles are armed with a specialized nematocyst population (den Hartog, 1977; Wellington, 1980) and inflict damage on neighboring colonies (Richardson *et al.*, 1979; Sheppard, 1979; Wellington, 1980; Chornesky, 1983). Certain acontiarian sea anemones display an analogous phenomenon. Following tentacle contact between adjacent anemones, one or both individuals will differentiate catch (or 'killer') tentacles. Like sweeper tentacles, these are elongate, are heavily armed with a specialized nematocyst population (Calgren, 1929; Hand, 1955; Williams, 1975; Purcell, 1977; Watson and Mariscal, 1983), and are used to injure neighbors (Williams, 1975, 1980; Purcell, 1977; Purcell and Kitting, 1982; Watson and Mariscal, 1983). Certain endomyarian sea anemones possess acrorhagia. These structures can inflate and, upon

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[1] Present address: Department of Zoology, University of Washington, Seattle, Washington 98195.

[2] Present address: Portland Shrine Research Unit, Shriners Hospital for Crippled Children, 3101 S. W. Sam Jackson Park Road, Portland, Oregon 97201.

contact with the adversary, discharge nematocysts (Abel, 1954; Bonnin, 1964; Frances, 1973b; Bigger, 1976, 1980; Williams, 1978; Ottaway, 1978; Brace and Pavey, 1978; Brace, *et al.*, 1979; Brace, 1981).

The evolution of this diverse array of structures is necessarily predicated on the existence of some underlying system of historecognition. The ability to distinguish between isogeneic, allogeneic, and xenogeneic tissues has been demonstrated in certain scleractinians (Lang, 1971, 1973; Hildeman *et al.*, 1975, 1977a, b, 1980), actiniarians (Frances, 1973a, b, 1976; Purcell, 1977; Bigger, 1980; Brace, 1981), gorgonians (Theodor, 1970, 1976; Theodor and Senelar, 1975; Bigger and Runyan, 1979), and hydroids (Teisser, 1929; Schijfsma, 1939; Crowell, 1950; Hauenschild, 1954, 1956; Muller, 1964, 1967; Toth, 1967; Ivker, 1972; Gallien and Gouere, 1974; Tardent and Buhrer, 1982; Muller *et al.*, 1983). It is widely assumed that histocompatibility and deployment of the various effector systems are genetically based alternatives. This assumption is supported by the common observation that aggressive devices are deployed against allogeneic tissue, but not in response to isogeneic tissue (Schijfsma, 1939; Muller, 1964, 1967; Lang, 1971, 1973; Ivker, 1972; Francis, 1973a, b, 1976; Theodor, 1976; Purcell, 1977; Bigger, 1980; Brace, 1981; Tardent and Buhrer, 1982). Genetic data, however, are available for only one cnidarian, the hydractiniid hydroid *Hydractinia echinata* (Hauenschild, 1954, 1956; Ivker, 1972).

Unlike anthozoans, for which there exist substantial data on the manner in which destruction of foreign tissue is effected, there is little comparable information for hydrozoans. Although instances of interspecific and intraspecific competition are known in several hydroid species (*e.g.*, Kato *et al.*, 1962, 1963, 1967; Chiba and Kato, 1966; Muller *et al.*, 1983), structures specialized for competition have been described only for members of the family Hydractinidae. In *H. echinata,* fusion was first noted by Teisser (1929) between planulae derived from the same cross. Ten years later, Schijfsma (1939) noted that fusion was not the only outcome of intraspecific encounters, noting that "it looks as if the growing borders of two colonies, in striking together and checking each others progress, are stimulated by very active growth and ramifications; resulting in the formation of a dense fringe of intertwined stolons." Subsequent studies by Crowell (1950), Hauenschild (1954, 1956), and Toth (1967) discussed the lack of compatibility between colonies but did not record the behavior of tissues in contact. Muller (1964), however, reported the presence of regions of "wild" stolonal growth in contact with incompatible tissues, observing that such growth may be initiated by both of the colonies in contact. He further observed that these modified stolons were associated with the regression and subsequent demise of one of the interacting colonies and suggested that this regression is due to a toxin released by the modified stolons. Ivker (1972) expanded on Muller's observations, introducing the term "hyperplastic stolon" to describe the modification of normal stolonal growth upon contact with foreign tissue. She likewise found that hyperplastic stolons destroy foreign tissue and hypothesizes that this destruction is the result of an enzymatic secretion from hyperplastic tissue. Subsequent studies of another hydractiniid, *Podocoryne carnea,* have documented a similar hyperplastic response to allogeneic (Tardent and Buhrer, 1982) and xenogeneic tissues (Gallien and Gouere, 1974).

In attempt to elucidate the mechanism by which hydractiniid hydroids effect the destruction of foreign tissues, we initiated a study of the fusion–rejection interaction in *H. echinata.* We find (a) that mat and stolonal tissues differ in their capacity to mount a hyperplastic response, (b) that production of hyperplastic tissue is dependent on differentiation of interstitial cells, and (c) that hyperplastic tissues effect their destruction of foreign tissue by nematocyst discharge. Comparison of anthozoan and

hydrozoan responses to foreign tissues suggests the need to distinguish between the selective forces responsible for the evolution of mechanisms of interference competition and those responsible for the evolution of historecognition.

Materials and Methods

Animal collection, maintenance and propagation

We report on a series of laboratory investigations on the phenomenology, ultrastructure, and mechanism of the histocompatibility response in *Hydractinia echinata*. Methods for each topic considered here are described in separate sections below. Common to all studies, however, are the source of experimental animals and our methods of cultivation and asexual propagation.

Hydractinia echinata grows as an encrustation on the surface of gastropod shells occupied by pagurid hermit crabs (Fig. 1). The colonies of *H. echinata* used in this study were collected on a shallow subtidal (<5 m) gravel-mud bottom at Harrison Point, Long Island Sound, from shells occupied by *Pagurus longicarpus*. Colonies collected from these shells are assumed to be isogeneic. This assumption is justified because asexual propagation from one shell to another is unknown and several different attempts to detect naturally occurring chimeras have failed (McFadden *et al.*, 1984).

Field-collected colonies were propagated by removing with a scalpel an explant of basal mat containing 1–3 feeding polyps from a shell and gently holding it to a plexiglass slide with a loop of suture thread. After 1–3 days explants attached and threads were removed. Stock colonies established in this manner were maintained in laboratory culture for a period of 2–14 months prior to this study. Colonies were maintained in a recirculating sea water system at room temperature and were fed with one-day-old brine shrimp nauplii for two hours daily. Explants from isogeneic stock colonies were attached to various experimental substrata (detailed below) for observations of colony ontogeny and histocompatibility interactions. Techniques of explantation and laboratory cultivation have been described in further detail elsewhere (Ivker, 1972; McFadden *et al.*, 1984).

Colony ontogeny, potential tissue interactions, and histocompatibility

Colonies of *H. echinata* vary considerably in gross morphology during early ontogeny (Schijfsma, 1939; Hauenschild, 1954; Ivker, 1972; McFadden *et al.*, 1984). The relative rates of production of mat, stolon, and polyps throughout ontogeny differ among colonies, producing a characteristic pattern in gross morphology for a given colony. Mat tissue is composed of a close network of entodermal gastrovascular canals surrounded by interstitial cells and covered by a uniform layer of ectoderm. Stolons are individual periderm-covered canals, composed of a layer of endoderm and a layer of ectoderm, which branch and anastomose to form a highly complex network criss-crossing the substratum. Feeding polyps arise from the mat (Fig. 1), and in some genotypes, from the stolons. Depending on the morphology of colonies and/or the time in ontogeny at which contact is made, there are three possible classes of interactions between isogeneic or allogeneic colonies: (1) mat contacting mat, (2) mat contacting stolon, and (3) stolon contacting stolon.

To insure observation of all possible tissue interactions, five genotypes of *H. echinata* were chosen. The ontogeny of each colony was quantified by observing the number of polyps, the area of mat, and the area of enclosed stolon through time by the methods of McFadden *et al.* (1984). No replicates were made of these observations, as explants from a given clone produce nearly identical patterns of colony ontogeny

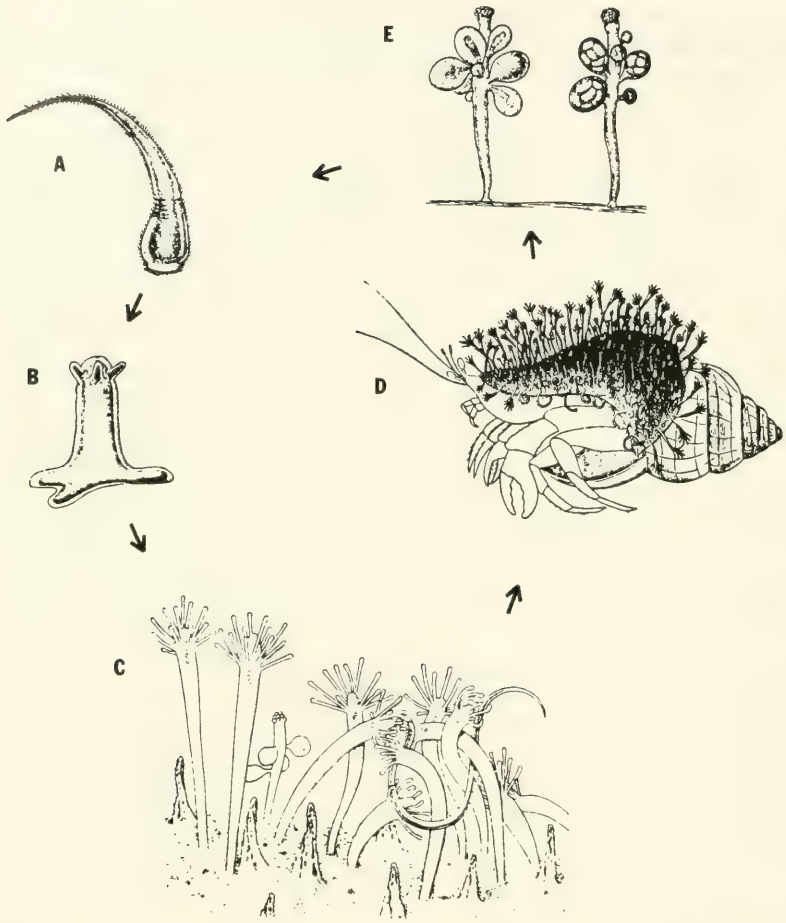


FIGURE 1. Life cycle of *Hydractinia echinata*. Fertilized egg develops into crawling planuloid larvae (A) which attaches to a substratum and metamorphoses into a primary polyp (B). By asexual iteration, this polyp develops into a mature colony (C, D) which will produce either male or female reproductive polyps (E). (from McFadden *et al.*, 1984).

(Buss and Grosberg, unpub.). Knowledge of the ontogenetic patterns allowed pairing of colonies at points in ontogeny such that all possible tissue interactions were observed in both isogeneic and allogeneic combinations. Each pairwise combination was replicated at least five times. Observations were made on the sequence of events following contact between colonies at 50× using a dissecting microscope.

Ultrastructure of the fusion–rejection interaction

Three categories of response to contact between colonies were noted using light microscopy: fusion, rejection with hyperplastic stolon formation, and rejection without hyperplastic stolon formation. The development of each of these three outcomes was examined using transmission electron microscopy. Explants of the appropriate colonies were attached to Lux petri dishes and fixed at various times after the initial contact between colonies. Colonies were fixed in modified Karnovsky's fixative (Karnovsky,

1965) containing 2% paraformaldehyde, 2.5% gluteraldehyde, 1.5 *M* $CaCl_2$ in 0.1 *M* final concentration sodium cacodylate buffer, pH 7.4, for two hours on ice, rinsed in buffer, then postfixed in 1% OsO_4 on 0.1 *M* sodium cacodylate buffer for one hour on ice. Colonies were then rinsed in buffer, dehydrated through a graded series of ethanol dilutions, treated with propylene oxide, infiltrated, and flat-embedded in their original Lux permanox petri dishes in Polybed 812 polymerized at 60°C overnight. Colonies were separated from the dishes, cut out with a jewelers saw, and either (1) mounted onto a blank for face-on sectioning across histocompatibility interactions or (2) clamped directly into a LKB Huxley ultramicrotome for cross-sectioning. Areas of isogeneic and allogeneic tissue interactions were located via light microscopy by examining 1 μ thick sections stained in 0.25% Azure I and 0.25% Azure II in 0.25% Sodium Borate. Once located, ultrathin sections from silver to light gold interference color were cut with a diamond knife and mounted on formvar coated 1 × 2 mm slot grids, allowing direct correlation of both the thick section via light microscopy and the entire thin section via transmission electron microscopy. Following staining in 2% Uranyl acetate in 50% Ethanol for 15 minutes and Reynold's lead citrate for 60 seconds, sections were examined and photographed using either a Philips E.M. 200 or Philips E.M. 300 operated at 60 kV.

The development of hyperplastic stolons was also observed in scanning electron microscopy, to help correlate transmission microscopy results with observations made with the dissecting microscope. Colonies were grown on glass cover slips and fixed by the same protocol as those prepared for transmission electron microscopy. Following dehydration through a graded series of ethanol, samples were taken through critical point in liquid CO_2 in a Sorvall critical point drying apparatus, and sputter coated with 60% Au, 40% Pd. Samples on coverslips were examined and photographed using an ETEC autoscan scanning electron microscope operated at 5–10 kV.

Interstitial cells and the development of hyperplastic stolons

Colonies were experimentally deprived of interstitial cells (I-cells) to assess the potential influence of the induced differentiation of nematocytes in histocompatibility interactions. The I-cells of hydroids appear to be a multipotent stem cell line, capable of differentiating into any of the various somatic cell types (Lentz, 1966; Muller, 1967, 1968). In the growing colony, however, I-cells only replace those cells incapable of mitotic activity: the nematocytes, the sensory-motor-interneurons, and the gametes (Diehl and Burnett, 1964, 1965a, b; Muller, 1964, 1967, 1968; Campbell and David, 1974; David and Murphy, 1977; Marcum and Campbell, 1978). In *H. echinata,* interstitial cells (I-cells) are located between gastrovascular canals within the mat and occur only rarely in the stolons (Muller, 1964).

Muller (1967, 1968) has demonstrated that application of mitomycin-C leads to the selective lysis of interstitial cells in *H. echinata.* Mitomycin-C acts primarily by attacking RNA synthesis and may secondarily lead to structural damage in DNA (Muller, 1967). Application of mitomycin-C leaves cnidoblasts, nerve cells, and epitheliomuscular cells intact and thus is preferable to the irradiation or nitrogen mustard techniques typically used with *Hydra* (Muller, 1967). Colonies exposed to mitomycin retain the ability to regenerate, produce new polyps, and elongate stolons. Treated colonies, however, can no longer differentiate nematocytes and will eventually die unless fed manually.

We experimentally eliminated the I-cell population of colonies to determine the capacity of I-cell-depleted organisms to recognize incompatible tissues and to mount a hyperplastic response. Three large colonies were exposed for 14 hours to 0.06 *M*

mitomycin-C. Immediately following the mitomycin exposure, four explants from an isogeneic, but unexposed colony were placed into contact with one of the exposed colonies to determine whether the I-cell-depleted colony retained its fusibility characteristics and, if so, to repopulate the depleted colony with I-cells. After two weeks, four explants from this exposed-replenished colony were placed in contact with allogeneic tissue as controls for the exposure process. The second exposed colony was used to test the capacity of an I-cell-depleted colony to mount a hyperplastic response. Eleven explants of allogeneic tissues were placed in contact with the exposed colony and observations made on the behavior of stolons in contact. The third colony was left unmanipulated and died within three weeks, indicating that the I-cell population of the colony had been effectively eliminated.

Results

Colony ontogeny and histocompatibility

The growth of polyps, mat, and stolon throughout ontogeny for the five genotypes are presented in Figure 2. The five strains differ significantly in the rate of growth of mat (ANOVA, $F = 4.49$, $P < 0.01$), polyps (ANOVA, $F = 3.03$, $P < 0.05$), and stolonal tissues (ANOVA, $F = 5.58$, $P < 0.005$). Log-transformed regressions of mat, polyp, and stolon tissues through time are presented in Table I. Inspection of Figure 2 illustrates that the five strains fall into three distinct groups. Strains 1 and 2 produce no stolons at any point in ontogeny, 4 and 5 produce stolons throughout ontogeny, and strain 3 only produces stolons late in ontogeny.

The histocompatibility responses of *H. echinata* were assessed in all paired combinations of the five strains (Fig. 3). In addition, strain 3 was paired with all other strains during both its early stolonless stage and late stoloniferous stage of ontogeny. Intraspecific contacts resulted in one of three unambiguous results: fusion, rejection without hyperplastic tissue formation, and rejection with hyperplastic tissue formation (Table II). Fusion is recognized by the disappearance of a discrete margin between tissues and the formation of a shared gastrovascular canal system (Fig. 4A). Rejection without hyperplasticity is recognized as the persistence of a discrete margin separating tissues in contact, with no evidence of shared gastrovascular systems (Fig. 4B). Rejection with hyperplasticity is recognized as the presence of swollen, erect stoloniferous tissues differentiating along, and extending atop, the contact zone (Fig. 4C, D).

Three relationships emerge from the results of paired histocompatibility interactions. First, all isogeneic combinations fuse and all allogeneic combinations reject (Table II). Second, fusion occurs in isogeneic crosses irrespective of the tissues which contact; whereas the pattern in rejection is dependent on the types of tissue which contact (Table II). Finally, mat and stolon tissue differ in their morphogenetic potential; only stolon can produce hyperplastic tissue. In allogeneic crosses, hyperplastic stolon is induced whenever stolons contact either foreign mat or stolon. Rejection without induction of hyperplasticity occurs only when foreign mats contact (Table II). It is important to note that strain 3 produced hyperplastic stolons in late ontogenetic encounters (*i.e.,* stolon-mat contacts) and failed to do so in early ontogenetic encounters (*i.e.,* mat-mat contacts), indicating that the different behavior of mat and stolonal tissues in histocompatibility interactions is purely a difference in the morphogenetic potential of the two tissue types.

Ultrastructure of fusion and rejection response

Contact between isogeneic tissues results in clear and unambiguous fusion between colonies of *H. echinata.* Fusion is recognized as the narrowing and rapid disappearance

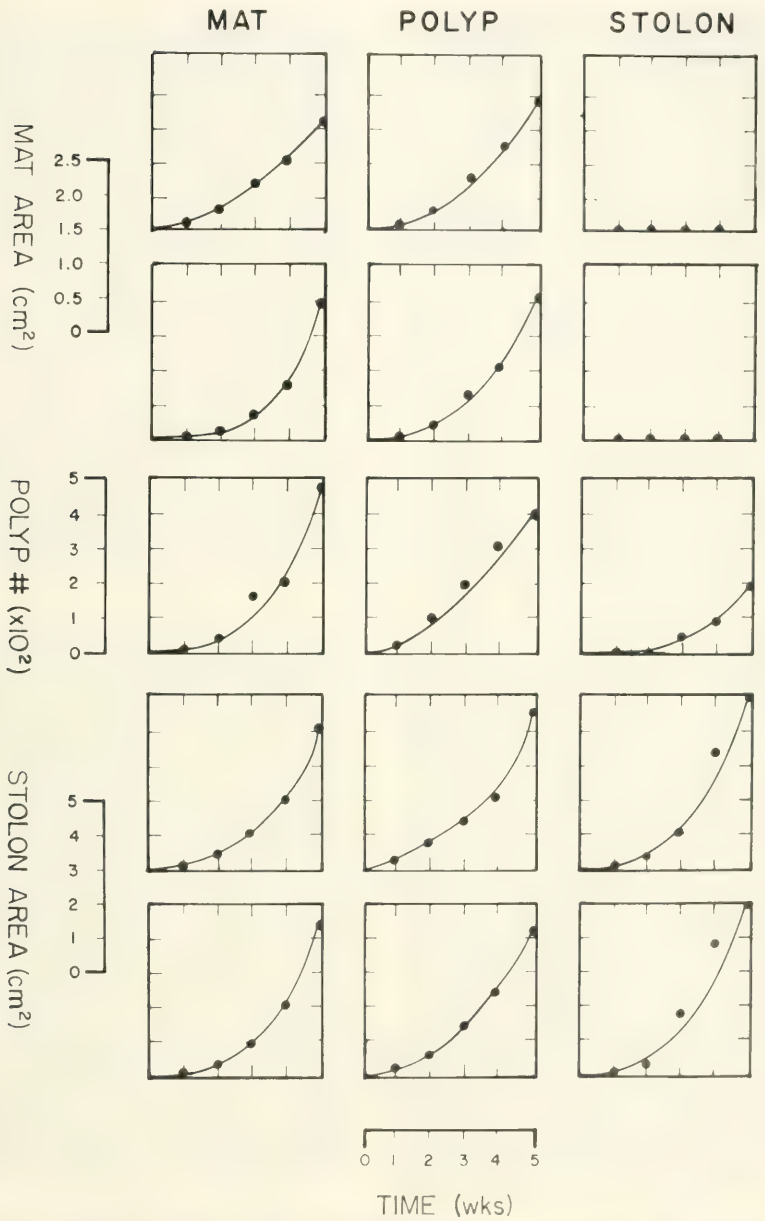


FIGURE 2. Colony ontogeny of the five genotypes of *Hydractinia echinata* used in studies of histocompatibility. Each row represents the growth for one genotype of mat area in cm^2, of the number of polyps, and the enclosed area of stolons in cm^2 *versus* time. Data for strains 1–5 appear sequentially in row order from top to bottom. Scales are the same for each plot.

of the periderm coat in the region of contact immediately following contact between colonies. Ultrastructural observations show no evidence of any boundary between cells of the two colonies as early as 1.5 hours following the initial contact (Fig. 5a). Within four hours of the initial contact a shared gastrovascular system has become

TABLE I

Colony ontogeny

Strain	Slope-Mat[1]	R^2	Signif.[2]	Slope-Polyp[1]	R^2	Signif.[2]	Slope-Stolon[1]	R^2	Signif.[2]
1	0.398	.957	$P < 0.001$	0.424	.987	$P < 0.001$	—	—	—
2	0.568	.988	$P < 0.001$	0.397	.999	$P < 0.001$	—	—	—
3	0.512	.982	$P < 0.001$	0.587	.993	$P < 0.001$	.227	.836	$P < 0.05$
4	0.549	.990	$P < 0.001$	0.734	.948	$P < 0.001$	.387	.963	$P < 0.001$
5	0.402	.993	$P < 0.001$	0.481	.936	$P < 0.001$	.301	.969	$P < 0.001$

[1] Log (mat, stolon, polyp) *versus* log (time).
[2] F-test.

established, as evidenced in live observations by the movement of granular material from one colony into the other.

Rejection between allogeneic tissues is characterized by a distinct fibrous boundary separating the two colonies (Fig. 5b, c). This fibrous boundary appears distinct from the periderm coat, is secreted by both colonies, and occurs in both types of rejection responses. At no point have we seen any direct cell-to-cell contact between colonies, nor any evidence of either cells or vesicles crossing this boundary. It is important,

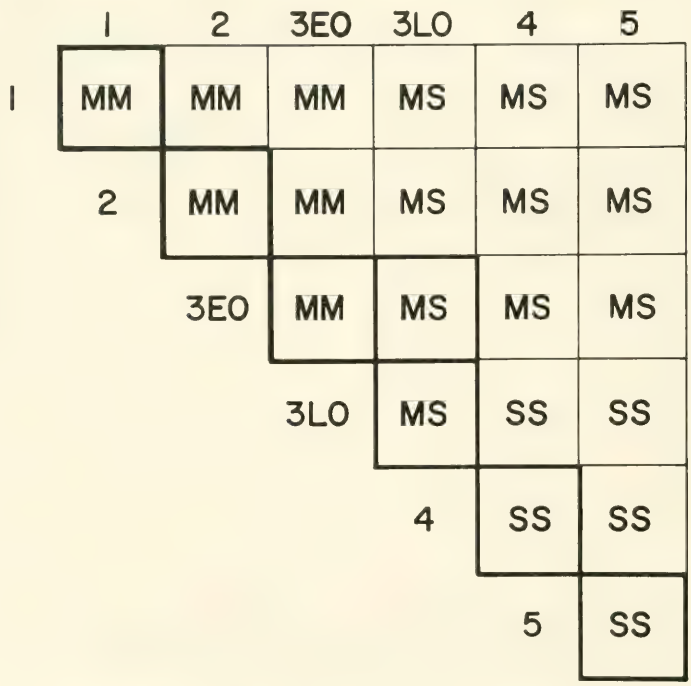


FIGURE 3. Matrix of the tissue interactions resulting from combinations of the five genotypes. Columns and rows represent strain numbers. Note that strain 3 was tested at two different times during ontogeny, during its early ontogenetic (3EO) stolonless phase and its late ontogeny (3LO) stoloniferous stage. Bold face cells represent isogeneic combinations, all other cells represent allogeneic interactions. Five replicates were made for each cell in this matrix. MM-mat *versus* mat interactions, MS-mat *versus* stolon interactions, and SS-stolon *versus* stolon interactions.

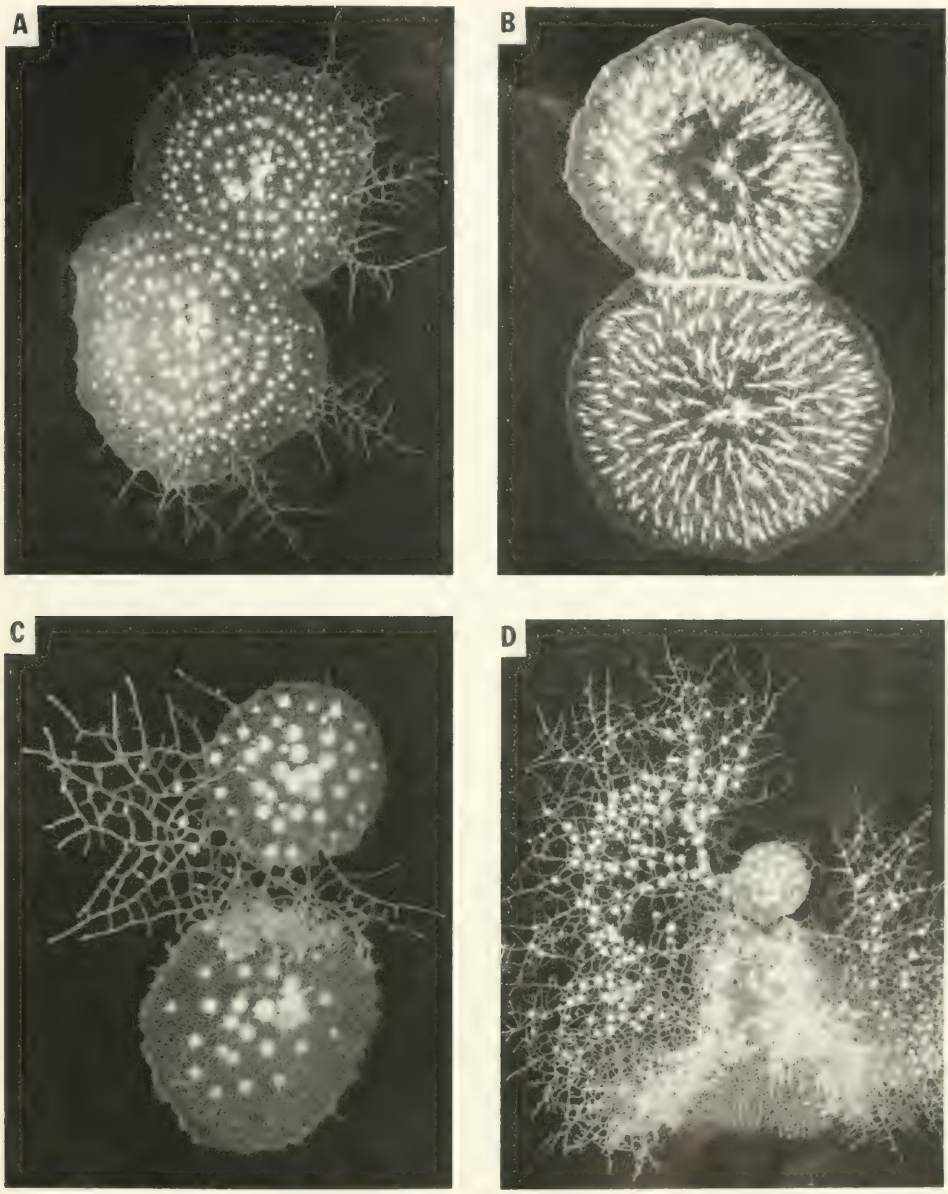


FIGURE 4. (A) Fusion between two colonies of *Hydractinia echinata*. Note the continuous gastrovascular canals traversing the margin between colonies. (B) Rejection between mats of two incompatible colonies. Note the failure to fuse along shared colony margin. (C) Rejection between a stolon producing colony and a colony which produces no stolons. Note development of hyperplastic stolons where stolons contact the mat of the foreign colony. (D) Rejection between two stolon producing strains, showing hyperplastic stolon development where stolons of the two colonies contact.

however, to recognize that microvillar extensions of ectodermal cells frequently perforate the mucous layer, hence direct cell surface communication is not ruled out by our observations.

TABLE II

Histocompatibility interactions

Tissues in Contact	n	Fusion	Rejection*	
			No Hyperplastic Response	Hyperplastic Response
A. Isogeneic Interactions				
Mat *versus* Mat	15	15	0	0
Mat *versus* Stolon	10	10	0	0
Stolon *versus* Stolon	10	10	0	0
B. Allogeneic Interactions				
Mat *versus* Mat	15	0	15–15	0–0
Mat *versus* Stolon	40	0	40–0	0–40
Stolon *versus* Stolon	15	0	0–0	15–15

* First figure represents behavior of first tissue type listed.

Rejection by mat and stolonal tissues differs fundamentally in that stolonal tissues undergo a complex series of morphogenetic transitions following contact with foreign tissue. Within 24 hours of the original contact, stolons become markedly swollen and begin to lose their periderm coat. These swollen or hyperplastic stolons lift up off the substratum and begin to redirect growth toward the foreign colony (Fig. 7a). Upon contacting the foreign tissue, the tissues underlying the stolon lyse. At the ultrastructural level, this series of events is recognized as the movement of numerous cnidoblasts and interstitial cells into the stolon, the development of a distinctive cnidom on the surface of the hyperplastic stolon coming into contact with the foreign tissue (Fig. 6a, b), and the discharge of numerous nematocysts of the basotrichious isorhizal type (Fig. 7c; Mariscal, 1974) into the foreign tissues and the associated lysis of cells in the region of contact (Fig. 6c, d, 7b).

Rejection in I-cell-depleted colonies

I-cell-depleted colonies retain their fusibility characteristics, fusing with isogeneic colonies (n = 4) and failing to fuse with allogeneic tissues (n = 11). I-cell-depleted colonies, however, failed to display a typical hyperplastic response. Upon contacting foreign tissue, stolons of I-cell-depleted colonies swelled very slightly. These stolons, however, failed to continue to swell in the typical fashion or to lift off the substratum and redirect growth toward the foreign colony. Exposed colonies with their I-cell population replenished (n = 4) displayed a wholly typical hyperplastic response to allogeneic tissues. These experiments demonstrate that the induction of hyperplasticity is dependent upon I-cells, but that the recognition of foreign tissue upon initial contact between colonies is not.

DISCUSSION

The hyperplastic response of *H. echinata* to allogeneic tissue bears a number of similarities to anthozoan responses to neighbors. Both hydrozoan and anthozoan responses (1) require contact for induction; (2) are capable of discriminating between

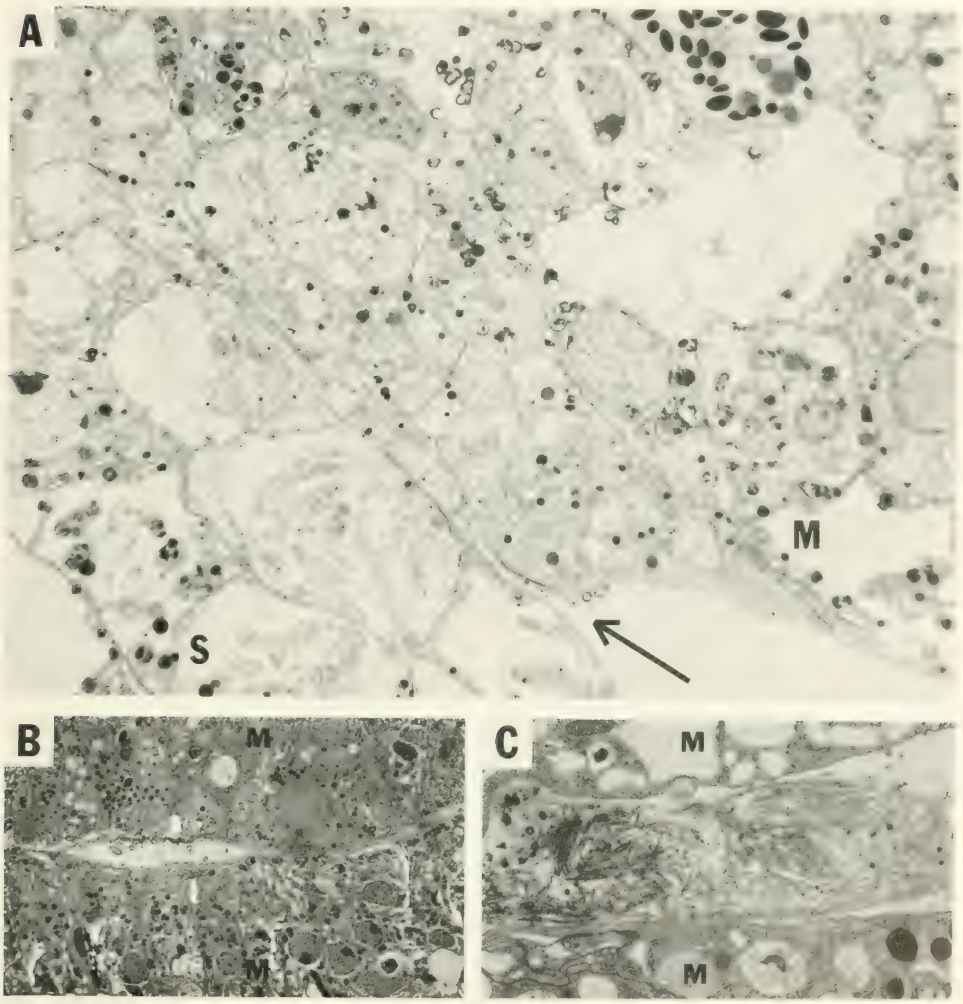
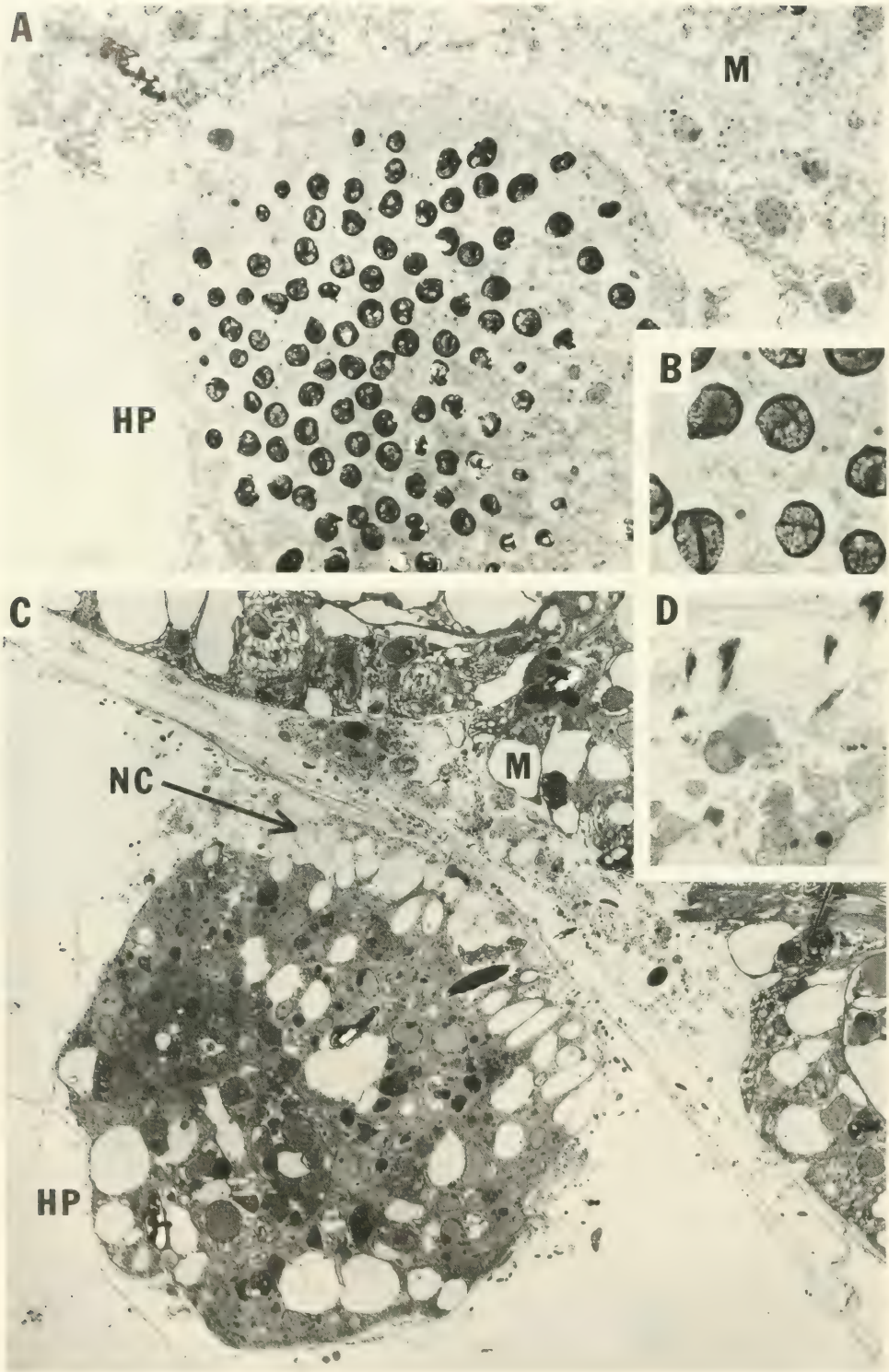


FIGURE 5. (A) Fusion of mat and stolonal tissues 1.5 hours after initial contact between colonies (1390×). Arrow points to region of initial contact. Note the lack of any distinct boundary separating cells of the two colonies. (B) Rejection between mats of two allogeneic colonies (1200×). Lying between the two colonies, along the entire length of boundary, is an electron-dense fibrous material. This fibrous layer, shown at higher magnification (8040×) in (C), is not in contact with the tissues of either colony. M = mat, S = stolon.

isogeneic and allogeneic tissues; (3) respond by site-specific cellular differentiation; and (4) involve the discharge of nematocysts to effect destruction of foreign tissues.

Recognition elements

Anthozoan responses to foreign tissues are apparently elicited by contact with either the tentacles, coenosarcs, or mesenterial filaments of other cnidarians. In *H. echinata,* the response is elicited following contact with either mat or stolonal tissue. Cnidarians are typically covered with a copious mucous layer, perforated with microvillar extensions of ectodermal cells. Tardent and Buhrer (1982) suggest that rec-


A
M
HP
B
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NC
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HP

ognition elements lie within the mucous layer of *Podocoryne carnea,* but they do not consider the possible influence of cell-surface markers on ectodermal villi. Bigger (1976), however, tested the capacity of allogeneic mucus to elicit an acrorhagial response in *Anthopleura krebsi* and found no such effect. Lubbock (1979) demonstrated that mucous extractions of various sea anemone and coral species have markedly different antigenic determinants. He failed, however, to detect differing antigenic determinants in mucus within a given species. The localization and eventual characterization of recognition elements remains a central, unresolved issue.

Historecognition

A hallmark of anthozoan responses to neighboring cnidarians is the capacity to distinguish between isogeneic and allogeneic tissues and to selectively deploy effector systems against allogeneic forms. To my knowledge, all substratum-bound cnidarians investigated are able to distinguish between isogeneic and allogeneic tissues (Table III). In contrast to the apparent uniformity of allorecognition, cnidarian recognition of xenogeneic tissues is quite variable. Several anemones fail to display acrorhagial responses upon interspecific encounters with other anemones (Francis, 1973; Bigger, 1976, 1980; Williams, 1978), despite the ability of at least one anemone to recognize tissues as different as that of a scyphozoan medusae (Bigger, 1976, 1980). Similarly, catch tentacle development in *Metridium senile* may vary greatly in both occurrence and effect on other anemones (Purcell and Kitting, 1982). Among scleractinians, sweeper tentacles in *Agaricia agaricities* may develop in response to encounters with the encrusting gorgonian *Erythropodium caribaeoreum* and the zooanthid *Palythoa caribbea* (Chornesky, 1983). Similarly, the hydrocoral *Millepora dichotoma* displays varying degrees of interspecific aggression in response to xenogeneic neighbors (Muller *et al.,* 1983).

The apparent ubiquity of allorecognition may reflect a primitive capability of cnidarians and the variability in deployment of effector systems in xenogeneic encounters may be a relatively recent adaptation to local circumstances. If this hypothesis is correct, xenogeneic effector systems should be found most frequently between species in which the frequency and potential severity of interspecific encounters is great. This suggestion is tentatively supported by observations of the interactions among hydractiniid hydroids in Long Island Sound. *Hydractinia echinata* is the most common hydractiniid and interactions are primarily intraspecific contacts, whereas *Podocoryne carnea* is relatively rare and makes frequent interspecific encounters (Buss and Yund, unpub.). As expected, *P. carnea* is capable of mounting a sustained hyperplastic response to *H. echinata,* whereas *H. echinata* is incapable of maintaining a similar response to *P. carnea* (McFadden, unpub.). Further study of the relationship between the occurrence of xenogeneic effector systems and the relative frequency of intraspecific and interspecific competition is warranted.

FIGURE 6. (A) Section across the tip of a hyperplastic stolon in contact with foreign mat (800×). Note high density of nematocysts in hyperplastic stolon. 96 hours after initial contact between colonies. (B) Inset of this cross-section in higher magnification (3273×), shows that each cell harbors a nematocyst. (C) Section across hyperplastic stolon in contact with foreign mat (800×). Note the concentration of capsules of discharged nematocysts along the margin of the hyperplastic stolon where it is in contact with foreign tissue and the zone of destruction directly underlying this region. These discharged capsules are eventually sloughed off, a new set of nematocytes are differentiated, and the interaction repeated until the foreign tissue is completely eliminated. (D) Inset shows the contact zone at greater magnification (3273×), showing shafts of the nematocysts embedded in the foreign tissue. HP = hyperplastic stolon, M = mat, NC = nematocyst capsule.

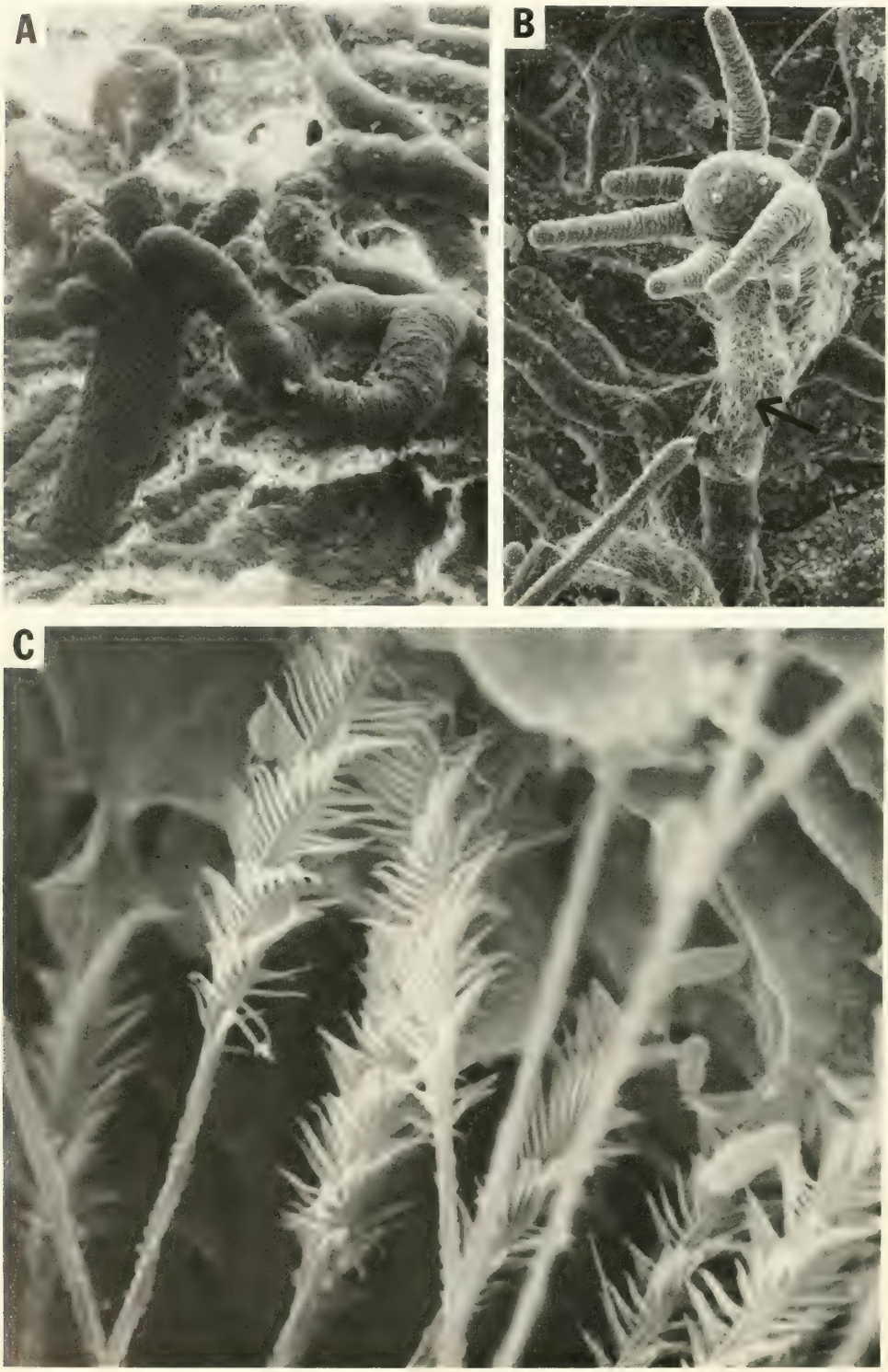
A
B
C

TABLE III

Cnidarian histocompatibility and competition

Taxon	Effector System	References
Hydrozoa		
Hydroida		
Hydractinia echinata	Hyperplastic stolons	Schijfsma, 1939; Muller, 1964; Ivker, 1972
Podocaryne carnea	Hyperplastic stolons	Tardent and Buhrer, 1982
Milleporina		
Millepora dichotoma	Unknown	Muller *et al.*, 1983
Anthozoa		
Gorgonacea		
Lophogorgia sarmentosa	Unknown	Theodor, 1970
Eunicella stricta	Unknown	Theodor, 1976
Leptogorgia virgulata	Unknown	Bigger and Runyan, 1979
Psuedopterogorgia elisabethae	Unknown	Bigger and Runyan, 1979
Plexaura flexuosa	Unknown	Bigger and Runyan, 1979
Actiniaria		
Actinea equina	Acrorhagi	Francis, 1973b, Brace and Pavey, 1978
Anthopleura artemisia	Acrorhagi	Bigger, 1980
A. balloii	Acrorhagi	Williams, 1978
A. elegantissima	Acrorhagi	Francis, 1973b
A. Krebsi	Acrorhagi	Bigger, 1976, 1980
Anemonia sargassensis	Acrorhagi	Bigger, 1980
Bunodosoma cauernata	Acrorhagi	Bigger, 1980
Phymactis clematis	Acrorhagi	Brace, 1981
Cereus pendunculatus	Catch Tentacles	Williams, 1975
Diadumene cincta	Catch Tentacles	Williams, 1975
Halipanella luciae	Catch Tentacles	Williams, 1975; Watson and Mariscal, 1983
Metridium senile	Catch Tentacles	Purcell, 1977
Sargartia elegans	Catch Tentacles	Williams, 1975
S. troglodytes	Catch Tentacles	Williams, 1975
Scleractinia		
Agaricia agaricites	Sweeper Tentacles	Chornesky, 1983
Montastrea cavernosa	Sweeper Tentacles	Richardson *et al.*, 1979
Montipora verrucosa	Unknown	Hildeman *et al.*, 1975, 1980
Pocillopora damicornis	Sweeper Tentacles	Wellington, 1980
P. robusta	Sweeper Tentacles	Wellington, 1980

Site-specific differentiation

The occurrence of such a diverse array of responses to foreign tissues testifies to the chronic occurrence of intra- and interspecific competition in cnidarians. Contacts between cnidarians are typically site-specific; interactions among scleractinians and

FIGURE 7. (A) Scanning electron micrograph showing a hyperplastic stolon arching off the substratum toward a polyp of an allogeneic colony (120×). (B) Contact between a hyperplastic stolon (arrow) and a foreign polyp (190×). Note the concentration of nematocysts threads where hyperplastic stolon contacts the foreign polyp. (C) Artificially discharged nematocysts from a hyperplastic stolon (440×), showing these nematocysts to be basotrichious isorhizas.

hydrozoans are typically made only along colony margins and interactions between anemones are often limited to only a portion of a clonal patch. Several cnidarian responses to foreign tissues (*e.g.*, sweeper tentacles, catch tentacles, hyperplastic stolons) share a common feature: the capacity for site-specific differentiation of specialized tissues and morphologies. The capacity for site-specific differentiation is enormously important as it allows a colony to divert energies to aggression only in those tissues where they may be most effective. Site-specific differentiation, however, can only occur if the group is capable of transporting multipotent stem cells (or their products) to the zone of combat. This trait is limited in phyletic distribution; only sponges, cnidarians, platyhelminthes, echinoderms, and chordates have been found to possess a mitotically active multipotent stem line throughout ontogeny (Nieuwkoop and Sutasurya, 1981; Buss, 1983a, b).

The dependence of several effector systems on site-specific differentiation underscores the need for caution in the interpretation of immunologic "memory" in invertebrates. The repeated reports of memory in invertebrates involve systems in which the effector mechanisms are unknown (Hildeman, 1975, 1977a, b, 1980; Manning, 1980; Bigger *et al.*, 1982). However, if these responses require differentiation of multipotent stem cells the observation of memory may simply reflect the deployment of specialized cells or cell products the differentiation of which had been previously induced. Although this will result in an accelerated second-set response, this observation does not imply that (a) the putative memory will be retained over ecologically relevant time scales or that (b) the accelerated second-set response will be observed to display any specificity whatsoever with respect to antigenic determinants. In the absence of a detailed knowledge of the nature of the effector system and appropriate third party experiments, the observation of an accelerated second-set response cannot be considered evidence of existence of a memory component homologous to that of vertebrate immune systems.

Effector systems

Perhaps the most striking similarity between the various groups of cnidarian responses to foreign tissues is the evolution of a nematocyst-based effector system. Nematocyst function is remarkable in its evolutionary lability; various specialized nematocysts are used for attachment, prey immobilization, prey capture, and clone defense (Mariscal, 1974). Nematocysts appear in structures as different and as limited in phyletic distribution as scleractinian sweeper tentacles and mesenterial filaments (den Hartog, 1977; Wellington, 1980), actinarian catch tentacles (Calgren, 1929; Hand, 1955; Williams, 1975; Purcell, 1977; Watson and Mariscal, 1982) and acrorhagia (Calgren, 1949; Abel, 1954; Bonnin, 1964; Francis, 1973b), and hydroid hyperplastic stolons (Figs. 6, 7). The use of nematocysts in histocompatibility and competition is likely a convergence in function.

Evolution of histocompatibility

The similarity of anthozoan and hydrozoan responses to foreign tissue suggests the need to distinguish between selection for histocompatibility and selection for competitive ability. Several authors have suggested that competition between individuals (or species) was the primitive selective agent shaping the evolution of allorecognition (*e.g.*, Kaye and Ortiz, 1981). This hypothesis seems unlikely for two reasons. It is difficult to understand how a diversity of different competitive behaviors and structures could have evolved if there were not a pre-existing system allowing for the recognition of those individuals and species against which they might be effective. In addition, cnidarians are uniformly capable of recognizing allogeneic tissues,

even in forms in which competition between conspecifics seems highly unlikely. A more parsimonious explanation is that genes for historecognition and totipotent cells capable of differentiating into nematocysts were ancestral features of cnidarians which became linked into certain groups. The diversity of cnidarian responses to competition may ultimately reflect the co-occurrence in this group of (1) a primitive system of historecognition, (2) a mitotically active multipotent stem cell lineage, and (3) an effective device, the nematocyst, which might be coopted to defensive functions. If this is the case, selective forces other than competition between individuals must account for the evolution of historecognition.

A frequently cited alternative explanation for the evolution of histocompatibility is that of defense against microbial and viral infections, cancer, and pathogen mimicry ("surveillence theory," *e.g.,* Burnet, 1970). Although microbial infections are undoubtedly of considerable importance, there is little data upon which to assess this theory in cnidarians. Allorecognition might, for example, be interpreted as a defense against the potential of fusion acting as a vector for pathogens. However, nematocyst-based effector systems are clearly unsuitable for employment against pathogens. Nematocysts are an order of magnitude larger than microbes and their unique method of deployment is clearly unrelated to any microbial clearance function. Cnidarians, however, may not be limited to nematocyst-based effector systems. For example, certain classes of cellular (Hildemann, 1975, 1977a, b) or allelochemic (Sammarco *et al.,* 1983) interactions have been suggested. Adequate assessment of the relevance of the "surveillence theory" to allorecognition in cnidarians must await further information on their mechanisms of microbial detection and clearance.

An alternative, but complementary, explanation for the evolution of histocompatibility is the somatic cell parasitism hypothesis (Buss, 1982). This hypothesis is based on the fact that the primordial germ cells of certain simple metazoans are not sequestered in early ontogeny. Fusion between conspecifics results in the passage of totipotent cells (*i.e.,* competent to produce gametes) from one individual into another. If the totipotent cells of one individual prove more effective in differentiating into gametes than do those of the other component of the chimera, then one individual has effectively parasitized the other (Buss, 1982). Fusion between individuals with an active totipotent cell lineage produces a chimera in which the fitness of the components of the chimera is determined not only by the fitness of the chimeric individual relative to other individuals in the population, but also by competition between components of the chimera for representation in the gametes. Systems of allorecognition serve to prevent fusion and the subsequent invasion of the totipotent cell line of one individual into another, hence acting to defend an organism from somatic cell parasitism. If this scenario is correct, the totipotency of cell lines provides both the *raison d'etre* for the evolution of historecognition and the mechanism permitting the subsequent evolution of specialized competitive mechanisms in the Cnidaria.

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LITERATURE CITED

ABEL, E. F. 1954. Ein Beitrag zur Giftwirkung der Actinien und Function der Randsackchen. *Zool. Anz.* **153:** 259–268.

BIGGER, C. H. 1976. The acrorhagial response in *Anthopleura krebsi:* intraspecific and interspecific recognition. Pp. 127–136 in *Coelenterate Ecology and Behavior,* G. O. Mackie, ed. Plenum Press, New York.

BIGGER, C. H. 1980. Interspecific and intraspecific acrorhagial aggressive behavior among sea anemones: a recognition of self and not-self. *Biol. Bull.* **159:** 117–134.

BIGGER, C. H., AND RUNYAN, R. 1979. An *in situ* demonstration of self-recognition in gorgonians. *Dev. Comp. Immnol.* **3:** 591–597.

BIGGER, C. H., P. L. JOKIEL, W. H. HILDEMAN, AND I. S. JOHNSTON. 1982. Characterization of alloimmune memory in a sponge. *J. Immunol.* **129:** 1570–1572.

BONNIN, J. P. 1964. Recherches sur la 'reaction d'aggression' et sur le fonctionnement des acorhages d'*Actinia equina* L. *Bull. Biol. Fr. Belg.* **1:** 225–250.

BRACE, R. C. 1981. Intraspecific aggression in the colour morphs of the anemone *Phymactis clematis* from Chili. *Mar. Biol.* **64:** 85–93.

BRACE, R. C., AND J. PAVEY. 1978. Size-dependent dominance hierarchy in the anemone *Actinia equina. Nature* **273:** 752–753.

BRACE, R. C., J. PAVEY, AND D. L. J. QUICKIE. 1979. Intraspecific aggression in the color morphs of the anemone *Actinia equina:* the convention governing dominance ranking. *Anim. Behav.* **27:** 553–561.

BURNET, M. 1970. *Self and Not-Self.* Cambridge Univ. Press, Cambridge. 318 pp.

BUSS, L. W. 1982. Somatic cell parasitism and the evolution of somatic tissue compatibility. *Proc. Natl. Acad. Sci. U.S.A.* **79:** 5337–5341.

BUSS, L. W. 1983a. Evolution, development and the units of selection. *Proc. Natl. Acad. Sci. U.S.A.* **80:** 1387–1391.

BUSS, L. W. 1983b. Somatic variation and evolution. *Paleobiology.* **9:** 12–16.

CALGREN, O. 1929. Uber eine Actiniariengattung mit besonderen Fangtentakeln. *Zool. Anz.* **81:** 109–113.

CAMPBELL, R. D., AND DAVID, C. N. 1974. Cell cycle kinetics and development of *Hydra attenuata.* II. Interstitial cells. *J. Cell. Sci.* **16:** 349–358.

CHIBA, Y., AND M. KATO. 1966. Interspecific relation in the colony formation among *Bougainvillia* sp. and *Cladonema radiatum* (Hydrozoa, Coelenterata). *Sci. Rep. Tohoku Univ. Ser. IV (Biol.)* **32:** 201–206.

CHORNESKY, E. A. 1983. Induced development of sweeper tentacles on the reef coral *Agaricia agaricites:* a response to direct competition. *Biol. Bull.* **165:** 569–581.

CROWELL, S. 1950. Individual specificity in the fusion of hydroid stolons and the relationship between stolonic growth and colony growth. *Anat. Rec.* **108:** 560–561.

DAVID, C. N., AND S. MURPHY. 1977. Characteristics of interstitial stem cells in *Hydra. Dev. Biol.* **58:** 372–383.

DIEHL, F. A., AND A. BURNETT. 1964. The role of interstitial cells in the maintenance of hydra. I. Specific destruction of interstitial cells in normal, asexual, non-budding animals. *J. Exp. Zool.* **155:** 253–260.

DIEHL, F. A., AND A. BURNETT. 1965a. The role of interstitial cells in the maintenance of hydra. II. Budding. *J. Exp. Zool.* **158:** 283–298.

DIEHL, F. A., AND A. BURNETT. 1965b. The role of interstitial cells in the maintenance of hydra. III. Regeneration of hypostome and tentacles. *J. Exp. Zool.* **158:** 299–318.

FRANCIS, L. 1973a. Clone specific segregation in the sea anemone *Anthopleura elegantissima. Biol. Bull.* **144:** 64–72.

FRANCIS, L. 1973b. Interspecific aggression and its effects on the distribution of *Anthopleura elegantissima* and some related sea anemones. *Biol. Bull.* **144:** 73–92.

FRANCIS, L. 1976. Social organization within clones of the sea anemone *Anthopleura elegantissima. Biol. Bull.* **150:** 361–376.

GALLIEN, L., AND M. C. GOUERE. 1974. Incompatibilite entre cultures inergeneriques d'explants chez hydraires *Hydractinia echinata* Fleming et *Podocoryne carnea* Sars. *Comptes. r. hebd. Seane. Acad. Paris Ser. D.* **278:** 107–110.

GLYNN, P. W. 1976. Some physical and biological determinants of coral community structure in the eastern Pacific. *Ecol. Mongr.* **46:** 431–456.

HAND, C. 1955. The sea anemones of Central California, Part III. The acontiarian anemones. *Wasmann J. Biol.* **13:** 189–251.

DEN HARTOG, J. C. 1977. The marginal tentacles of *Rhodactis sanctithomae* (Corallimorpharia) and the sweeper tentacles of *Montastrea cavernosa* (Scleractinia); their cnidom and possible function. Pp. 463–469 in *Proc. Third. Int. Coral Reef Symp.,* D. L. Taylor, ed. Univ. of Miami Press, Coral Gables.

HAUENSCHILD, V. C. 1954. Genetische und entwichlungphysiologische Untersuchungen uber Intersexualitat und Gewebevertraglichkeit bei *Hydractinia echinata* Flem. *Wilhelm Roux Arch. Entwicklungsmech. Org.* **147:** 1–41.

HAUENSCHILD, V. C. 1956. Uber die Vererbung einer Gewebevertraglichkeits-Eigenschaft bei dem Hydroidpolypen *Hydractinia echinata. Z. Naturforsch.* **11b:** 132–138.

HILDEMAN, W. H., D. S. LINTHICUM, AND D. C. VANN. 1975. Transplantation and immunoincompatibility reactions among reef-building corals. *Immunogenetics* **2:** 269–284.

HILDEMAN, W. H., R. L. RAISON, G. CHEUNG, C. J. HULL, L. AKAKA, AND J. OKAMOTO. 1977a. Immunological specificity and memory in a scleractinian coral. *Nature* **270:** 219–223.

HILDEMAN, W. H., R. L. RAISON, C. J. HULL, L. AKAKA, J. OKUMOTO, AND G. CHEUNG. 1977b. Tissue transplantation immunity in corals. Pp. 537–543 in *Proc. Third Int. Coral Reef Symp.*, D. L. Taylor, ed. Univ. of Miami Press, Coral Gables.

HILDEMAN, W. H., P. L. JOKIEL, C. H. BIGGER, AND I. S. JOHNSTON. 1980. Allogeneic polymorphism and alloimmune memory in the coral, *Montipora verrucosa. Transplantation* **30:** 297–301.

IVKER, F. B. 1972. A hierarchy of histo-incompatibility in *Hydractinia echinata. Biol. Bull.* **143:** 162–174.

KARNOVSKY, M. J. 1965. A formaldehyde-gluteraldehyde fixative of high osmolality for use in electron microscopy. *J. Cell. Biol.* **27:** 137A–138A.

KATO, M., K. NAKAMURA, E. HIRAI, AND Y. KAKINUMA. 1962. Interspecific relation in the colony formation among some hydrozoan species. *Bull. Mar. Bio. St. Asamushi. Tohoku Univ.* **11:** 31–36.

KATO, M., E. HIRAI, AND Y. KAKINUMA. 1963. Further experiments on the interspecific relation in the colony formation among some hydrozoan species. *Sci. Rep. Tohoku Univ. Ser. IV (Biol.)* **29:** 317–325.

KATO, M., E. HIRAI, AND Y. KAKINUMA. 1967. Experiments on the coaction among hydrozoan species in the colony formation. *Sci. Rep. Tohoku Univ. Ser. IV (Biol.)* **33:** 359–373.

KAYE, H., AND T. ORTIZ. 1981. Strain specificity in a tropical marine sponge. *Mar. Biol.* **63:** 165–173.

LANG, J. C. 1971. Interspecific aggression by scleractinian corals I. the rediscovery of *Scolymia cubensis* (Milne Edwards and Haime). *Bull. Mar. Sci.* **21:** 952–959.

LANG, J. C. 1973. Interspecific aggression by scleractinian corals II. Why the race is not always to the swift. *Bull. Mar. Sci.* **23:** 260–279.

LENTZ, T. L. 1966. *The Cell Biology of Hydra.* North-Holland Publ. Co., Amsterdam. 199 pp.

LUBBOCK, R. 1979. Mucous antigenicity in sea anemones and corals. *Hydrobiologia.* **66:** 3–6.

MANNING, M. 1980. *Phylogeny of Immunological Memory.* Elsevier, New York. 359 pp.

MCFADDEN, C. S., M. MCFARLAND, AND L. W. BUSS. 1984. Biology of hydractiniid hydroids. I. Colony ontogeny in *Hydractinia echinata. Bio. Bull.* **166:** 54–67.

MARISCAL, R. N. 1974. Nematocysts. Pp. 129–178 in *Coelenterate Biology: Reviews and Perspectives*, L. Muscatine and H. M. Lenhoff, eds. Academic Press, New York.

MARCUM, B. A., AND R. D. CAMPBELL. 1978. Development of *Hydra* lacking nerve and interstitial cells. *J. Cell Sci.* **29:** 17–33.

MULLER, W. E. G. 1964. Experimentelle Untersuchungen uber Stockentwicklung, Polypendifferenzierung und sexual Chimaren bei *Hydractina echinata. Wilhelm Roux' Arch. Entwicklungsmech. Org.* **155:** 181–268.

MULLER, W. E. G. 1967. Differenzierungspotenzen und Geschlechtstabilitat der I-Zellen von *Hydractinia echinata. Wilhelm Roux Arch. Entwicklungsmech. Org.* **159:** 412–432.

MULLER, W. E. G. 1968. Elimination der I-Zellen durch alkylierende Cytostatika und deren Effekte auf die Embryonalentwicklung bei *Hydractinia echinata. Exp. Cell. Res.* **49:** 448–458.

MULLER, W. E. G., A. MAIDHOF, R. K. ZAHN, AND I. MULLER. 1983. Histocompatibility reactions in the hydrocoral *Millepora dichotoma. Coral Reefs* **1:** 237–241.

NIEUWKOOP, P. D., AND L. A. SUTASURYA. 1981. *Primordial Germ Cells in the Invertebrates.* Cambridge Univ. Press, Cambridge.

OTTAWAY, J. R. 1978. Population ecology of the intertidal anemone *Actinia tenebrosa.* I. Pedal locomotion and intraspecific aggression. *J. Mar. Freshwater Res.* **29:** 787–802.

PURCELL, J. E. 1977. Aggressive function and induced development of catch tentacles in the sea anemone *Metridium senile* (Coelenterata, Actiniaria). *Biol. Bull.* **153:** 355–368.

PURCELL, J. E., AND C. L. KITTING. 1982. Intraspecific aggression and population distribution of the sea anemone *Metridium senile. Biol. Bull.* **162:** 345–359.

REYNOLDS, E. S. 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *J. Cell. Biol.* **17:** 208–212.

RICHARDSON, C. A., P. DUSTAN, AND J. C. LANG. 1979. Maintenance of the living space by sweeper tentacles of *Montastrea cavernosa. Mar. Biol.* **55:** 181–186.

SAMMARCO, P. W., J. C. COLL, S. LABARRE, AND B. WILLIS. 1983. Competitive strategies of soft corals (Coelenterata:Octocorallia): allelopathic effects on selected scleractinian corals. *Coral Reefs* **1:** 173–178.

SCHIJFSMA, K. 1939. Preliminary notes on early stages in the growth of colonies of *Hydractinia echinata* (Flem.). *Arch. Neerland. Zool.* **4:** 93–102.

SHEPPARD, C. R. C. 1979. Interspecific aggression between reef corals with reference to their distribution. *Mar. Ecol. Prog. Ser.* **1:** 237–247.

TARDENT, P., AND M. BUHRER. 1982. Intraspecific tissue incompatibilities in the metagenetical *Podocoryne carnea* M. Sars (Cnidaria:Hydrozoa). Pp. 295–303 in *Embryonic Development, Part B. Cellular Aspects,* M. M. Burger and R. Weber, eds. A. R. Liss, New York.

TEISSIER, G. 1929. L'Origine multiple de certaines colonies d'*Hydractinia echinata* (Flem.) et ses consequences possibles. *Bull. Soc. Zool. Fr.* **54:** 645–647.

THEODOR, J. L. 1970. Distinction between 'self' and 'not-self' in lower invertebrates. *Nature* **227:** 690–692.

THEODOR, J. L. 1976. Histocompatibility in a natural population of gorgonians. *Zool. J. Linn. Soc.* **58:** 173–176.

THEODOR, J. L., AND R. SENELAR. 1975. Cytotoxic interaction between gorgonian explants: mode of action. *Cell. Immunol.* **19:** 194–200.

TOTH, S. E. 1967. Tissue compatibility in regenerating explant from the colonial marine hydroid *Hydractinia echinata. J. Cell Physiol.* **69:** 125–131.

WATSON, G. M., AND R. N. MARISCAL. 1983. The development of a sea anemone tentacle specialized for aggression: morphogenesis and regression of the catch tentacle of *Haliplanella luciae* (Cnidaria, Anthozoa). *Biol. Bull.* **164:** 506–517.

WELLINGTON, G. M. 1980. Reversal of digestive interactions between Pacific reef corals: mediation by sweeper tentacles. *Oecologia* **47:** 340–343.

WILLIAMS, R. B. 1975. Catch-tentacles in anemones: occurrence in *Haliplanella luciae* (Verill) and a review of current knowledge. *J. Nat. Hist.* **9:** 241–248.

WILLIAMS, R. B. 1978. Some recent observations on the acrorhagi of sea anemones. *J. Mar. Biol. Assoc. U.K.* **58:** 787–788.

WILLIAMS, R. B. 1980. A further note on catch tentacles in anemones. *Trans. Norfolk Norwich Nat. Soc.* **25:** 84–86.

Reference: *Biol Bull.* **167**: 159–167. (August, 1984)

DISPERSAL OF ZOOXANTHELLAE ON CORAL REEFS BY PREDATORS ON CNIDARIANS

GISÈLE MULLER PARKER*

Department of Biology, University of California, Los Angeles, California 90024

ABSTRACT

Fish and nudibranchs prey on cnidarians that contain high densities of symbiotic dinoflagellates (zooxanthellae). Several fish (*Arothron meleagris, Chaetodon auriga,* and *Chaetodon unimaculatus*) and one nudibranch (*Berghia major*) feed on the Hawaiian symbiotic sea anemone *Aiptasia pulchella.* Fecal material of these predators consisted primarily of zooxanthellae, which were shown to be photosynthetically active and capable of re-establishing symbioses with aposymbiotic *A. pulchella.*

INTRODUCTION

Symbiotic dinoflagellates (zooxanthellae = *Symbiodinium microadriaticum*) are major primary producers on coral reefs (Muscatine, 1980). They occur in high densities in corals and other cnidarian hosts but have yet to be found in abundance in the water column. Since zooxanthellae must be acquired *de novo* with each sexual generation in many symbioses (Trench, 1979), and since cnidarians show specificity for different strains of zooxanthellae (Schoenberg and Trench, 1980), the question often arises: how are zooxanthellae dispersed over coral reefs? There are several ways in which zooxanthellae are freed from animal tissue: by extrusion, either spontaneous (Steele, 1977) or as a result of osmotic (Goreau, 1964) or temperature (Buchsbaum, 1968) stress, or as a result of predation on the host. Motile stages also can arise from zooxanthellae in decaying host nudibranch tissues (Kempf, 1984). The potential significance of predator feces as an agent for the dispersal of viable zooxanthellae has not been previously reported.

Many predators on corals and other symbiotic cnidarians have been described. For example, fishes of the Marshall Islands include browsers on coral polyps (Families: Chaetodontidae, Monacanthidae), grazers on coral heads (F.: Chaetodontidae, Scaridae, Balistidae, Monacanthidae), and feeders on branching coral tips (F.: Balistidae, Monacanthidae, Tetraodontidae, Canthigasteridae) (Hiatt and Strasburg, 1960; see also Hobson, 1974; Randall, 1974). Robertson (1970) reviewed invertebrate feeders on corals, particularly gastropods. However, none of these authors considered whether zooxanthellae were digested by the predators. The liberation of zooxanthellae from host tissue has two important ecological consequences: zooxanthellae are dispersed and may reinfect other hosts, and they become available as a food source for herbivores.

The Hawaiian sea anemone *Aiptasia pulchella* is found in shallow (<2 m) reef areas and in protected lagoons. The puffer *Arothron meleagris,* two species of butterflyfish, *Chaetodon auriga* and *C. unimaculatus,* and the nudibranch *Berghia major* were found to feed on *A. pulchella.* As each sea anemone contained from 1.0 to 1.5 $\times 10^7$ zooxanthellae, or about 3.0×10^6 zooxanthellae per mg animal protein (Parker, in prep.), large numbers of zooxanthellae were consumed. This paper presents data which show that fecal pellets from these predators contained stages which were pho-

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* Current address: School of Life Sciences, University of Nebraska, Lincoln, Nebraska 68588.

tosynthetically active and gave rise to motile zooxanthellae. Fecal zooxanthellae were also capable of re-establishing symbioses with aposymbiotic *A. pulchella;* this indicates that predator feces may be important as a mode for the dispersal of symbionts and reinfection of symbiotic hosts.

Materials and Methods

Feeding experiments

Experiments with *Arothron meleagris* were conducted at the University of California, Los Angeles, using fish and *Aiptasia pulchella* collected in Hawaii. Those with butterflyfishes and nudibranchs were conducted at the Hawaii Institute of Marine Biology (HIMB), Coconut Island, Kaneohe Bay, Oahu, Hawaii, using freshly collected organisms. Butterflyfishes were collected on the reefs near HIMB with baited live traps. The nudibranch was found among populations of *A. pulchella* near the docks at HIMB.

In controlled experiments I allowed predators which had been starved for 24 hours to feed on sea anemones, then isolated them in tanks of clean sea water. Fecal material was collected with a pipette in some cases within minutes, but usually within several hours after defecation.

Photosynthetic ability of fecal zooxanthellae

The photosynthetic ability of fecal zooxanthellae from *Arothron meleagris* was tested by fixation of ^{14}C-bicarbonate. Feces were suspended in sea water, briefly homogenized with a glass tissue homogenizer, and filtered through coarse "nitex" screen to remove large clumps. The filtrate was centrifuged at $300 \times g$ in an IEC HN-S table centrifuge for two minutes to separate zooxanthellae from debris. The algal pellet was resuspended in filtered sea water, assayed for cell number, and diluted to a concentration of 2.90×10^5 zooxanthellae $\cdot$ ml^{-1}. Cells were incubated with $NaH^{14}CO_3$ (0.5 μCi $\cdot$ ml^{-1}) in duplicate test tubes at an irradiance of 100 μE $\cdot$ m^{-2} $\cdot$ s^{-1} at 25°C for one hour. Replicate tubes wrapped in foil were also incubated to correct for any heterotrophic fixation of ^{14}C-bicarbonate. Tubes were inverted every 15 minutes to resuspend settled cells.

Photosynthesis by fecal zooxanthellae was compared with that of freshly isolated zooxanthellae. The latter were obtained from sea anemones maintained in the dark for periods corresponding to the residence times of consumed zooxanthellae in fish guts. Zooxanthellae were isolated as follows. Sea anemones were homogenized using a glass tissue homogenizer. The homogenate was centrifuged at $550 \times g$ to separate animal and algal fractions and the resulting algal pellet was washed in filtered sea water several times. Final suspensions were diluted to the same cell concentration used for fecal zooxanthellae, and cells were incubated with ^{14}C-bicarbonate under the same conditions used for the fecal zooxanthellae and at the same time. At the end of the incubations, tubes of fecal and freshly isolated zooxanthellae were centrifuged at $550 \times g$, and the resulting algal pellets were rinsed three times in filtered sea water. The supernatant and final algal volumes were recorded, and three replicate 100 μl aliquots were taken from each fraction for liquid scintillation counting. The aliquots were acidified with an equal volume of 1 *N* HCl and placed under a heat lamp for three hours to drive off inorganic $^{14}CO_2$, then neutralized by addition of 1 *N* NaOH. Scintillation fluor (10 ml) was added and samples were counted on a Beckman LS 100C scintillation counter. CPM were converted to DPM by the external standard ratio method.

Reinfection experiments with aposymbiotic A. pulchella

To determine if fecal zooxanthellae from all four predators could re-establish symbioses with aposymbiotic sea anemones, two containers of aposymbiotic *A. pulchella* (with four sea anemones per container) were set up for each source of feces. Feces were added directly to one container. The other container served as a control for spontaneous reinfection. Both containers in each of the four sets were aerated and sea anemones were maintained under the same irradiance and fed every other day with freshly hatched *Artemia* nauplii. After four days the sea water was changed in both containers and feces were removed from the experimental container. Containers were then rinsed with fresh sea water every two days. One or two tentacles were removed every two days from all sea anemones, squashed, and examined microscopically for the presence of zooxanthellae. The number of zooxanthellae and the day of first appearance were recorded.

To determine the extent of reinfection after a long period of time, the photosynthetic abilities of experimental and control sea anemones were compared after 50 days by measuring oxygen flux and fixation of ^{14}C-bicarbonate. Oxygen flux measurements were made in a rectangular plexiglas chamber (volume: 49.5 ml) with a Clark YSI 4004 oxygen electrode connected to a chart recorder. The chamber was surrounded by a water jacket maintained at 25°C. Irradiance sufficient for light-saturated photosynthesis by zooxanthellae in these sea anemones, 2000 $\mu E \cdot m^{-2} \cdot s^{-1}$, was provided by a 250 watt tungsten-halogen lamp. Several measurements in the light and dark were made for each sea anemone. Sea anemones were then incubated with 0.25 μCi $NaH^{14}CO_3$ per ml for one hour at 2000 $\mu E \cdot m^{-2} \cdot s^{-1}$ irradiance and 25°C. At the end of the incubations they were rinsed in non-radioactive sea water, homogenized, and the homogenate sampled for liquid scintillation counting, zooxanthellae cell numbers, and total protein. Aliquots for liquid scintillation counting were treated as previously described. To determine the density of zooxanthellae in experimental and control sea anemones, the homogenates were centrifuged to separate animal and algal fractions and the resulting algal pellet was washed three times with filtered sea water. Cell counts were made on the final algal suspensions. Supernatants were combined and aliquots analyzed for animal protein. Protein analysis was done by the method of Lowry *et al.* (1951). The density of zooxanthellae was expressed as numbers of zooxanthellae per mg animal protein.

Statistics

To determine whether the results of the reinfection experiments were significantly different for control and experimental groups of sea anemones, I used the Mann-Whitney U test (Sokal and Rohlf, 1969). Nonparametric statistics were necessary as data sets were found to be heteroscedastic (F_{max}-test; Sokal and Rohlf, 1969).

Results

Feeding experiments

A. pulchella was fed to puffers (*Arothron meleagris*) previously maintained on fresh mussel meat and occasionally sea anemones. Puffers curled back their lips and used their fused beak-like teeth to chop off the crown of tentacles from individual sea anemones. After the tentacles were consumed the puffer would eat the rest of the body column before proceeding to the next sea anemone. A puffer ate 15 to 45 sea anemones at one feeding. Defecation of sea anemone remains occurred 12 to 46 hours afterwards.

Individuals of *A. pulchella* from natural populations living on dead coral skeletons and rocks were offered to many different reef fishes in Hawaii. The butterflyfishes *Chaetodon auriga* and *C. unimaculatus* readily ate these sea anemones, with each fish consuming about five sea anemones at one feeding. The tentacle crowns and upper parts of the body column of sea anemones were preferred, and defecation occurred within 12 to 24 hours after feeding.

The nudibranch *Berghia major* was found in association with natural populations of *A. pulchella* in Hawaii. It may be that *A. pulchella* is a significant prey item for *B. major,* as nudibranchs laid egg strings close to these sea anemones. The nudibranch limited its feeding to sea anemone tentacles. One nudibranch consumed the tentacles of three sea anemones (approx. 120 tentacles) daily. Tentacles were clipped off near the oral disk. Nudibranchs defecated within 24 hours after feeding. *B. major* differs from the fish in that it stores zooxanthellae and nematocysts in its cerata. Zooxanthellae stored in the cerata are presumed to be photosynthetically active to some extent, as nudibranchs consumed less oxygen in the light than in the dark (Parker, unpubl.).

Fecal material from the three predatory fish and the nudibranch consisted mostly of zooxanthellae. Light microscopic examination of feces showed that fecal zooxanthellae appeared intact and that many of the cells were in the process of dividing (Fig. 1). Motile zooxanthellae arose within a few hours from defecated zooxanthellae when feces were placed under bright light.

Photosynthetic ability of fecal zooxanthellae

Photosynthetic rates of fecal zooxanthellae from the puffer *A. meleagris* are shown in Table I. Assimilation numbers, corrected for dark heterotrophic fixation, of fecal zooxanthellae are similar to those obtained for the freshly isolated zooxanthellae.

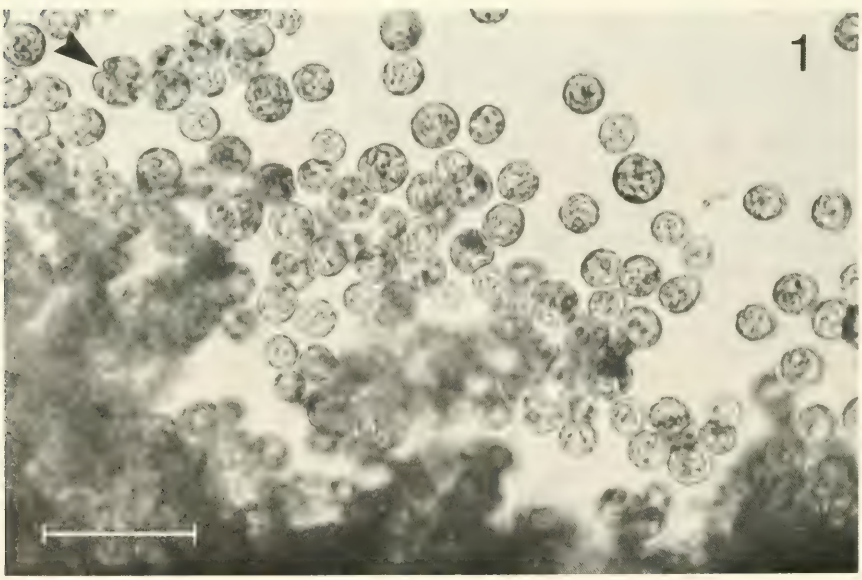

FIGURE 1. Light microscopic preparation of a fecal pellet from the nudibranch *Berghia major* which fed on the sea anemone *Aiptasia pulchella.* Arrow points to a dividing zooxanthella cell. Scale bar = 35 μm.

TABLE I

Assimilation numbers for zooxanthellae from Arothron meleagris *feces and freshly isolated from* Aiptasia pulchella

Sample	Fecal zooxanthellae: Time in fish gut (hours)	Fecal zooxanthellae: Assimilation number* (mg C · h^{-1} · zoox. $cell^{-1}$) ($\times 10^{-9}$)	Freshly isolated zooxanthellae: Time sea anemones kept in dark (hours)	Freshly isolated zooxanthellae: Assimilation number (mg C · h^{-1} · zoox. $cell^{-1}$) ($\times 10^{-9}$)
A†	12–24	3.30	0	2.32
A	24–36	0.02	36	0.77
B	24–36	2.65	46	1.55
C	46	0.94		

* Assimilation number $= \left[\left(\frac{\text{light } ^{14}C \text{ fixation} - \text{dark } ^{14}C \text{ fixation}}{\text{added activity}}\right) \cdot \left(\frac{0.09 \text{ mg } CO_2}{\text{ml}}\right) \cdot \left(\frac{\text{total volume}}{\text{incubation}}\right) \cdot \left(\frac{12C}{44\ CO_2}\right) \cdot h^{-1} \cdot (\text{total number of zooxanthellae})^{-1}\right]$

$= \text{mg C} \cdot \text{zooxanthella cell}^{-1} \cdot h^{-1}$.

† Letters refer to different fish individuals used.

These results indicate that fecal zooxanthellae are photosynthetically active after passage through the predator's gut.

Reinfection experiments with aposymbiotic A. pulchella

Within six to ten days after the initial addition of feces to containers of aposymbiotic *A. pulchella,* zooxanthellae, including some in division stages, appeared in all experimental sea anemone tentacles. Fecal zooxanthellae from all four predators reinfected aposymbiotic sea anemones. Control sea anemone tentacles remained initially free of zooxanthellae, although some contained zooxanthellae after 50 days in the light.

To determine the extent of reinfection after a long period of time, experimental and control sea anemones were compared at 50 days. The data in Table II show that after 50 days experimental sea anemones contained high densities of zooxanthellae. Significantly fewer zooxanthellae per mg animal protein were found in control sea anemones than in experimental ones which had been exposed to predator feces [Mann-Whitney U test: $U_s = 16$, $P < .05$ (for *C. unimaculatus* experiment) $U_s = 12$, $P < .10$ (for *B. major* experiment)].

The photosynthetic performance of control and experimental sea anemones after 50 days was evaluated from ^{14}C-bicarbonate fixation and oxygen production and consumption data (Table III). Significantly more carbon (DPM · mg sea anemone $protein^{-1}$) was fixed in experimental sea anemones than in control sea anemones [Mann-Whitney U test: $U_s = 16$, $P < .05$ (for *C. uminaculatus* experiment) $U_s = 12$, $P < .10$ (for *B. major* experiment)]. Experimental sea anemones showed net oxygen production in the light whereas control sea anemones consumed oxygen under the same conditions. Rates of oxygen consumption in the dark for both groups are included for comparison.

In separate experiments, fecal pellets were placed onto the oral disks of sea anemones to determine whether these were ingested. In most trials, feces were readily ingested and retained by both symbiotic and aposymbiotic sea anemones.

TABLE II

Zooxanthellae in experimental and control sea anemones 50 days after initial challenge

Donor predator (source of feces)	Recipient sea anemones	Total number of zooxanthellae per sea anemone ($\times 10^6$)	Number of zooxanthellae per mg animal protein ($\times 10^6$)
Chaetodon unimaculatus	Experimental (+ feces)	5.78 (±.28)† n = 4	2.88 (±.33) n = 4
	Control	0.08 (±.04) n = 4	0.05 (±.02) n = 4
Berghia major	Experimental (+ feces)	8.76 (±.78) n = 3	2.70 (±.02) n = 3
	Control	1.09* (±.84) n = 4	0.50 (±.38) n = 4

* One control sea anemone became densely packed with zooxanthellae. If it is excluded the mean number of zooxanthellae per sea anemone is 0.268×10^6.

† ±S.E.

DISCUSSION

Many symbiotic associations rely on a renewed establishment of the symbiosis after sexual reproduction (Trench, 1979). A few examples include the gorgonian *Pseudopterogorgia bipinnata* (Kinzie, 1974), the coral *Astrangia danae* (Szmant-Froelich *et al.*, 1980), the sea anemones *Anthopleura elegantissima* and *A. xanthogrammica* (Siebert, 1974), and the clam *Tridacna squamosa* (Fitt and Trench, 1981). The eggs and planula larvae of *Aiptasia pulchella* do not contain zooxanthellae (Parker, unpubl. obs.). As symbionts must be obtained from the environment, predator feces may be an important source of zooxanthellae for reinfection.

TABLE III

Productivity in experimental and control sea anemones 50 days after initial challenge

Donor predator (source of feces)	Recipient sea anemones	^{14}C fixed†	O_2 produced (+) or consumed (−)*	
			Light ($\times 10^{-2}$)	Dark ($\times 10^{-2}$)
Chaetodon unimaculatus	Experimental (+ feces)	53,302 (±8568)†† n = 4	+2.381 (±.538) n = 4	−1.003 (±.159) n = 4
	Control	2126 (±1057) n = 4	−0.411 (±.067) n = 3	−0.434 (±.143) n = 3
Berghia major	Experimental (+ feces)	131,600 (±33,533) n = 3	+2.327 (±.288) n = 3	−0.779 (±.221) n = 3
	Control	16,119 (±12,863) n = 4	−0.140 (±.253) n = 4	−0.809 (±.202) n = 4

* $(mg\ O_2) \cdot (mg\ sea\ anemone\ protein)^{-1} \cdot h^{-1}$.

† $DPM \cdot (mg\ sea\ anemone\ protein)^{-1}$.

†† ±S.E.

Although zooxanthellae are believed to be a single species, some strain specificity has been demonstrated in certain hosts (Schoenberg and Trench, 1980). Kinzie and Chee (1979) showed that aposymbiotic *A. pulchella* reinfected with zooxanthellae isolated from different hosts had different growth rates; sea anemones reinfected with zooxanthellae from *A. pulchella* and the scyphozoan *Cassiopea xamachana* grew as well as normal *A. pulchella* whereas those infected with zooxanthellae isolated from the gastropod *Melibe pilosa* and the clam *Tridacna maxima* grew no better than control aposymbiotic sea anemones. Therefore mechanisms which increase zooxanthellae dispersal, and thus contribute to the probability of host contact with the "correct" strain, are important. Mobile predators such as reef fish which release viable zooxanthellae in their feces may be significant in the dispersal of zooxanthellae over long distances.

Aposymbiotic sea anemones which had been exposed to feces contained high densities of zooxanthellae after 50 days (Table II). These densities were similar to those of symbiotic *A. pulchella,* which have algal densities ranging from 1.5 to 3×10^6 zooxanthellae per mg animal protein (Parker, in prep.). Although sea anemones exposed to fecal zooxanthellae had significantly more zooxanthellae, some of the control sea anemones became repopulated with zooxanthellae. Zooxanthellae in control sea anemones were probably residual cells, occasionally found in aposymbiotic *A. pulchella,* which multiplied under the favorable culture conditions.

Predation on symbiotic cnidarians may increase the chances of zooxanthellae coming into contact with other host organisms, as fecal pellets were found to be readily ingested by *A. pulchella.* Feces contain varying quantities of semi-digested animal remains which may stimulate ingestion in potential host organisms. It is not yet known if motile zooxanthellae released from the fecal material, direct ingestion of feces, or both processes, are responsible for reinfection of aposymbiotic hosts. Observations with cultured zooxanthellae indicate that the motile forms are more readily ingested by potential hosts than the non-motile zooxanthellae (Kinzie, 1974; Fitt and Trench, 1981), but predator feces consist of freshly isolated zooxanthellae associated with animal remains and hence may not be directly compared with cultured zooxanthellae. It is likely that both motile zooxanthellae from fecal pellets and direct ingestion of fecal pellets are modes for acquisition of symbionts.

Many zooxanthellae in the process of cell division were found in predator feces (Fig. 1). It is possible that passage through the predator gut may expose zooxanthellae to higher nutrient levels than are found in sea water. This may actually stimulate growth and the survival of fecal zooxanthellae, as has been shown for algae in the guts of freshwater *Daphnia magna* (Porter, 1976).

This study shows that zooxanthellae defecated by some predators on cnidarians are viable. The different assimilation numbers for fecal and freshly isolated zooxanthellae cannot be directly attributed to factors such as residence time in the fishes or dark preconditioning of host sea anemones (Table I). Variability in the photosynthetic performance of fecal zooxanthellae may result from differences in the physiological environment encountered by zooxanthellae during passage through the fish guts. There is a possibility that some of the consumed zooxanthellae were digested. Herbivorous reef fishes may break down plant material by mechanical grinding or lysis by gastric acidity (Lobel, 1981). All zooxanthellae in the feces appeared intact and healthy (for example, Fig. 1), suggesting that mechanical breakdown is negligible. Harmelin-Vivien and Bouchon-Navaro (1983) studied the diets of butterflyfishes in Moorea. They measured the ratio of the weight of the alimentary tract to the weight of the fish (defined as a repletion index) and found that diet was correlated to the repletion index. They found that chaetodontids feeding primarily on corals had a

greater proportion of their body weight as alimentary tract, and from this concluded that corals represent more of a vegetable food (*i.e.*, zooxanthellae) than an animal food for butterflyfishes. However, they did not examine the fecal material of these butterflyfishes, nor did they present any physiological evidence for this conclusion. Although some herbivorous reef fishes have acidic gastric fluids and plant material is degraded by these (Lobel, 1981), no data on the acidity of gastric fluids of butterflyfishes and puffers are available. There is no information on cellulase activity in the butterflyfishes and puffer used in this study, however two species of estuarine puffers from Georgia showed no cellulase activity (Stickney and Shumway, 1974). The results of this study indicate that at least a significant proportion of consumed zooxanthellae are not digested by the butterflyfishes and puffers.

The nudibranch differs from the fish in that zooxanthellae are selected and stored in the cerata. Since these were photosynthetically active in the nudibranch, zooxanthellae must have a process whereby digestion in *B. major* is avoided. It has been suggested that the nutritional status of nudibranchs may influence the relative proportion of degenerate and healthy zooxanthellae in fecal material (Kempf, 1984).

Fish feces have been shown to be a food source for other reef fish (Robertson, 1982), but the importance of zooxanthellae released from feces as a food source for coral reef filter-feeding herbivores is unknown. Large numbers of zooxanthellae are released in predator feces. As an example, a puffer weighing 270 g wet weight readily consumes 30 sea anemones at one feeding. One feeding may thus liberate up to 330 million zooxanthellae. The relative contribution of fecal zooxanthellae to reef food sources will depend on the density of predators and the amount of symbiotic tissue consumed and assimilated. Zooxanthellae in fish feces may be an important source of energy for the reef community as well as a source of zooxanthellae for the reinfection of nonsymbiotic larvae and juveniles of host species.

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LITERATURE CITED

BUCHSBAUM, V. 1968. Behavioral and physiological responses to light by the sea anemone *Anthopleura elegantissima* as related to its algal symbionts. Ph.D. Dissertation, Stanford University, Palo Alto, California.

FITT, W. K., AND R. K. TRENCH. 1981. Spawning, development, and acquisition of zooxanthellae by *Tridacna squamosa* (Mollusca, Bivalvia). *Biol. Bull.* **161:** 213–235.

GOREAU, T. F. 1964. Mass expulsion of zooxanthellae from Jamaican reef communities after Hurricane Flora. *Science* **145:** 383–386.

HARMELIN-VIVIEN, M. L., AND Y. BOUCHON-NAVARO. 1983. Feeding diets and significance of coral feeding among chaetodontid fishes in Moorea (French Polynesia). *Coral Reefs* **2:** 119–127.

HIATT, R. W., AND D. W. STRASBURG. 1960. Ecological relationships of the fish fauna on coral reefs of the Marshall Islands. *Ecol. Monogr.* **30:** 65–127.

HOBSON, E. S. 1974. Feeding relationships of teleostean fishes on coral reefs in Kona, Hawaii. *Fish. Bull.* **72:** 915–1031.

KEMPF, S. C. 1984. Symbiosis between the zooxanthella *Symbiodinium (= Gymnodinium) microadriaticum* (Freudenthal) and four species of nudibranchs. *Biol. Bull.* **166:** 110–126.

KINZIE, R. A. III. 1974. Experimental infection of aposymbiotic gorgonian polyps with zooxanthellae. *J. Exp. Mar. Biol. Ecol.* **15:** 335–345.

KINZIE, R. A. III, AND G. S. CHEE. 1979. The effect of different zooxanthellae on the growth of experimentally reinfected hosts. *Biol. Bull.* **156:** 315–327.

LOBEL, P. S. 1981. Trophic biology of herbivorous reef fishes: alimentary pH and digestive capabilities. *J. Fish Biol.* **19:** 365–397.

LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, AND R. J. RANDALL. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.

MUSCATINE, L. 1980. Productivity of zooxanthellae. Pp. 381–402 in *Primary Productivity in the Sea.* P. G. Falkowski, ed. Plenum Publishing Co., New York.

PORTER, K. G. 1976. Enhancement of algal growth and productivity by grazing zooplankton. *Science* **192:** 1332–1334.

RANDALL, J. E. 1974. The effect of fishes on coral reefs. *Proc. Second Int. Coral Reef Symp.* **1:** 159–166.

ROBERTSON, D. R. 1982. Fish feces as fish food on a Pacific coral reef. *Mar. Ecol. Prog. Ser.* **7:** 253–262.

ROBERTSON, R. 1970. Review of the predators and parasites of stony corals, with special reference to symbiotic prosobranch gastropods. *Pac. Sci.* **24:** 43–54.

SCHOENBERG, D. A., AND R. K. TRENCH. 1980. Genetic variation in *Symbiodinium (= Gymnodinium) microadiaticum* Freudenthal, and specificity in its symbiosis with marine invertebrates. III. Specificity and infectivity of *Symbiodinium microadriaticum. Proc. R. Soc. Lond. B.* **207:** 445–460.

SIEBERT, A. E., JR. 1974. A description of the embryology, larval development, and feeding of the sea anemones *Anthopleura elegantissima* and *A. xanthogrammica. Can. J. Zool.* **52:** 1383–1388.

STEELE, R. D. 1977. The significance of zooxanthella-containing pellets extruded by sea anemones. *Bull. Mar. Sci.* **27:** 591–594.

STICKNEY, R. R., AND S. E. SHUMWAY. 1974. Occurrence of cellulase activity in the stomachs of fishes. *J. Fish Biol.* **6:** 779–790.

SOKAL, R. R., AND F. J. ROHLF. 1969. *Biometry.* W. H. Freeman and Co., San Francisco. 776 pp.

SZMANT-FROELICH, A., P. YEVICH, AND M. E. Q. PILSON. 1980. Gametogenesis and early development of the temperate coral *Astrangia danae* (Anthozoa: Scleractinia). *Biol. Bull.* **158:** 257–269.

TRENCH, R. K. 1979. The cell biology of plant-animal symbiosis. *Ann. Rev. Plant Physiol.* **30:** 485–531.

Reference: *Biol Bull.* **167**: 168–175. (August, 1984)

MORPHOLOGICAL AND BEHAVIORAL DEFENSES OF TROCHOPHORE LARVAE OF *SABELLARIA CEMENTARIUM* (POLYCHAETA) AGAINST FOUR PLANKTONIC PREDATORS

J. TIMOTHY PENNINGTON AND FU-SHIANG CHIA

Department of Zoology, University of Alberta, Edmonton, Alberta, Canada T6G 2E9

ABSTRACT

Controlled experiments were conducted by offering eggs, pre-setal trochophores, and setose trochophores of the polychaete *Sabellaria cementarium* to four planktonic predators, *Pleurobrachia bachei* (Ctenophora), *Aequorea victoria* (Hydrozoa), brachyuran megalopa (Crustacea), and juvenile *Sebastes* spp. (Pisces). Each predator species captures prey with different mechanisms and the prey, while similar in size, differ in motility and presence or absence of setae.

Consumption of non-motile eggs was greater by megalopa but less by *A. victoria* than consumption of pre-setal trochophores; it is suggested that differences in predator feeding mechanisms account for these differences. Setose trochophores were always consumed at lower rates than the younger stages. The evidence suggests that setae can function in larval defense against an array of predators with different feeding mechanisms, but that swimming may increase, decrease, or have no effect upon rate of predation, depending upon predator species.

INTRODUCTION

Thorson (1946), Young and Chia (in press), and others have suggested that the major source of larval mortality for benthic marine invertebrates is predation. While this conjecture may be true, little empirical information supports it. Predation upon invertebrate larvae is generally documented during gut content analyses of predators; larvae usually constitute a minor portion of the diet (reviewed by Young and Chia, in press), and larvae thus observed are often partially digested and therefore difficult to identify. However, Cowden *et al.* (1984) provide data on differential predation upon several pelagic larvae by two benthic filter-feeders. Models of reproductive strategies of benthic invertebrates have generally assumed that rates of predation upon larvae are constant throughout ontogeny (Vance, 1973; Pechenik, 1979; Jackson and Strathmann, 1981), though Christiansen and Fenchel (1979) did consider large, late-stage larvae less susceptible to predation than small, early larvae.

Motility is a factor which may alter rates of predation upon developing larvae. Gerritsen and Strickler (1977) have predicted on the basis of encounter rates that prey could minimize predation by minimizing movement. However, it remains unclear whether diversity of planktivores and feeding mechanisms will render this hypothesis relatively unimportant in marine environments, especially for slow-swimming invertebrate larvae.

A second factor which may alter rates of larval predation is the development of structures such as larval setae (Fig. 1d). A wide variety of planktonic organisms develop setae or spines, including larvae of many benthic polychaetes (Bhaud and

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Cazaux, 1982; review by Schroeder and Hermans, 1975) and articulate brachiopods (Long, 1964). These larval setae project posteriorly during normal swimming, but are erected to spread out radially when larvae encounter objects or are otherwise disturbed (Fig. 1b–c). Since larval setae are typically lost during metamorphosis, they are presumed to be adaptations to pelagic existence. Setae and spines have been postulated to function both as "parachutes" which slow sinking rates and as defense mechanisms (Wilson, 1929, 1932; Hardy, 1956; Blake, 1969; Fauchald, 1974; Schroeder and Hermans, 1975). In defense, setae are presumed to function both by increasing a larva's effective size and by making it difficult to swallow. Spines of freshwater rotifers and cladocerans are known to be effective defenses against small plantivorous invertebrates, but are apparently not effective against fish predation (Gilbert, 1966; Dodson, 1974; Kerfoot, 1975, 1978, 1980). The only observations regarding the function of setae or spines for marine organisms are those of Lebour (1919) and Wilson (1929). Lebour (1919) observed a megalopa's dorsal spine lodging the larva into the esophagus of a small fish; the fish was neither able to expel or ingest it and eventually died. Wilson (1929) described small fish ejecting *Sabellaria alveolata* trochophores from their mouths and suggested that erected setae rendered the trochophores offensive.

This study was designed to examine whether motility and setae of trochophores of the polychaete *Sabellaria cementarium* Moore are effective defenses against predation by four planktonic predators. *S. cementarium* was used as prey because its embryos and larvae were readily available, and because of the prominent setae that its trochophores develop (Fig. 1b–d).

Materials and Methods

Adult *Sabellaria cementarium* were dredged in the vicinity of San Juan Island, Washington. Gametes were obtained and embryos and larvae were cultured as in Smith (1981). Non-motile eggs, 2 day-old pre-setal trochophores and 5 day-old setose trochophores were used as prey (Fig. 1a–c). Body size and shape was relatively constant during the first five days of development (70–90 μm), though eggs were disk-shaped and somewhat broader when freshly spawned.

Predator species from four phyla, *Pleurobrachia bachei* (Ctenophora), a medusa *Aequorea victoria* (Hydrozoa), unidentified brachyuran megalopa (Crustacea), and juvenile *Sebastes* sp. (Pisces), were chosen because they were common near Friday Harbor during summertime, and because of their different feeding mechanisms. Although in some cases predators were kept in the laboratory for several days before experiments and fed *Artemia salina* nauplii or goldfish food, they appeared to be in good condition at the time of experiments.

For each experiment fifty eggs or larvae were placed into each of 16 1.0 l jars which contained 960 ml of 3 μm filtered sea water. Twelve of the jars were divided into four sets of three replicates. Each set received a different predator species: (1) one 10 mm diameter *P. bachei* per jar; (2) one 30 mm diameter *A. victoria* per jar; (3) five 3 mm long megalopa per jar; or (4) two 15 mm long *Sebastes* sp. per jar. The four remaining jars served as controls, measuring background mortality and handling errors.

All jars were capped and strapped horizontally around the horizontal axis of a "grazing wheel" which rotated at 1.6 rpm, gently stirring the water and keeping the prey evenly distributed within the jars. Experiments were run for 24 hours in a 12:12 light:dark, 14°C coldroom. At the end of each experiment, predators were removed and water was siphoned from the jars through 41 μm Nitex mesh, concentrating the

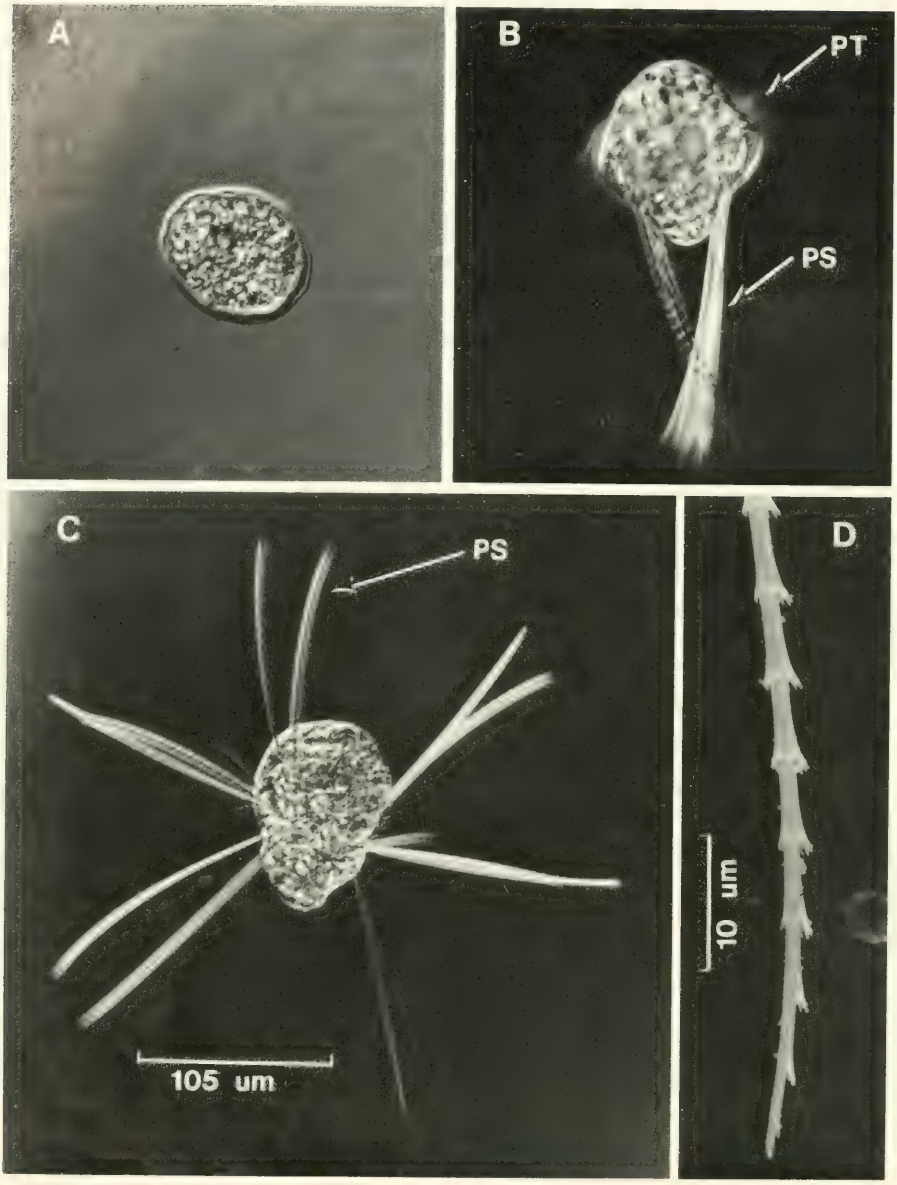

FIGURE 1. Selected developmental stages of *Sabellaria cementarium;* A, B, and C slightly compressed and to same scale. A: unhatched embryo of the same size and shape as eggs and pre-setal trochophores; B: five day-old setose trochophore swimming with unerected setae; C: five day-old trochophore with erected setae; D: seta of *S. cementarium* trochophore. PT, prototroch; PS, provisional setae.

remaining prey in a small volume of residual water. The prey were then washed into vials and preserved in 2% formalin. The preserved prey were later counted in a Bogorov Tray under a dissecting microscope.

Data analysis was performed according to the methods of Zar (1974).

Results

Predation rate upon the three developmental stages of *Sabellaria cementarium* by each of the four predators is presented in Figures 2a–d. All control values were averaged because loss from control jars was stage-independent; the slope of a least-squares regression of number of larvae missing from controls upon prey stage did not differ significantly from zero (F-test; $P < .05$). A one-way analysis of variance (ANOVA) was calculated from the data for each predator species to determine if there were significant differences between the number of prey missing in the four treatments (controls, eggs, pre-setal trochophores, and setose trochophores). The analyses were done with untransformed data since Bartlett's Test indicated that the data was sufficiently homoskedastic for ANOVA. For all ANOVA's there were significant overall differences between treatments ($P < .02$ or less), indicating that all predators ate some prey. *A posteriori* Student-Newman-Keuls Range Tests (SNK Tests) were then calculated which compared all possible combinations of treatments and grouped treatment subsets that were not significantly different ($P < .05$).

The different predator species exhibited different rates and patterns of predation upon eggs and pre-setal trochophores, but in all cases setose trochophores were eaten at low rates, not significantly different than control values (Fig. 2). For *Pleurobrachia bachei,* the SNK Test grouped values for the controls and setose trochophores as not different or homogeneous, indicating non-significant predation upon setose trochophores while eggs and pre-setal trochophores were eaten significantly more often.

For *Aequorea victoria* the SNK Test grouped values for the controls, eggs, and setose trochophores as homogeneous, indicating uniformly low rates of loss from

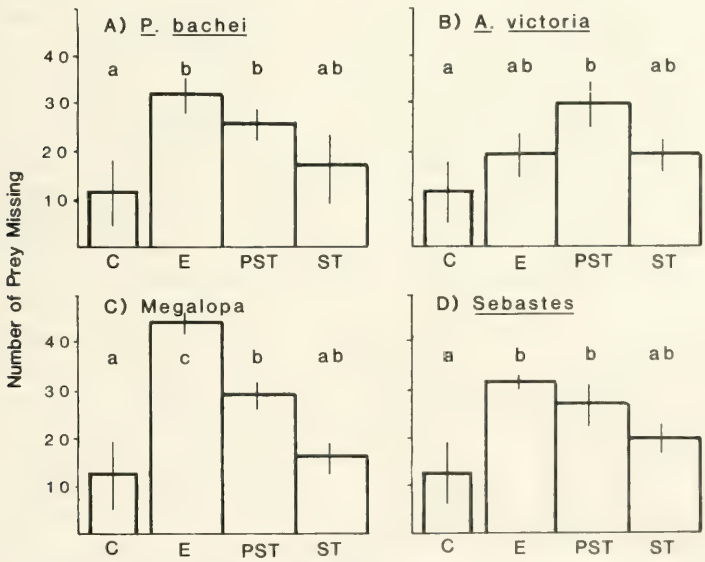

FIGURE 2. Histograms showing mean number of *Sabellaria cementarium* eggs and larvae missing from treatments for each of the four predator species, ± 1 standard deviation. Treatments are C, controls (n = 12); E, eggs (n = 3); PST, pre-setal trochophores (n = 3); ST, setose trochophores (n = 3). Letters over each bar denote the results of a Student-Newman-Keuls Multiple Range Test, where the same letter occurs over two or more bars the bars were grouped as not significantly different ($P < .05$).

these groups. Thus, pre-setal trochophores appear to be vulnerable to predation by *A. victoria,* but eggs and setose trochophores are either neglected or avoided.

The SNK Test indicated that brachyuran megalopa ate significantly more eggs than the other prey stages, but that insignificant numbers of setose trochophores were eaten.

For juvenile *Sebastes* the SNK Test again grouped the controls and setose trochophores as homogeneous, indicating that eggs and pre-setal trochophores combined were eaten significantly more often than setose trochophores.

Discussion

The effect of motility on predation rate varied among predators, probably a result of the predators' different feeding mechanisms. Predation by medusae involves responses to individual prey in the sense that nematocysts must be stimulated to fire, and prey motion is an important cue in this response (Pantin, 1942). Non-motility may explain the lack of consumption of eggs by *Aequorea victoria.* Prey motion is presumably an important cue for ctenophores and fishes as well, since colloblasts must be stimulated to release adhesive substance in ctenophores (Franc, 1978) and most fish locate prey visually (Kislalioglu and Gibson, 1976; Hyatt, 1979). However, *Pleurobrachia bachei* and *Sebastes* sp. did not eat more motile than nonmotile prey.

Megalopa ate significantly more eggs than all other stages of prey. It thus appears that swimming helped trochophores to escape or avoid these predators. The mechanisms by which most megalopa feed on small prey are not known, but many crustaceans both filter small particles and feed raptorially upon larger prey (Marshall and Orr, 1960; McLaughlin, 1982). If the megalopa did filter-feed, prey capture was probably not dependent upon recognition of individual eggs or trochophores. If so, non-motile eggs would be encountered and captured nearly as often as swimming trochophores, but if swimming enabled some trochophores to escape, the rate of predation upon eggs would be higher, as was observed.

Predation upon setose trochophores was insignificant while oocytes and pre-setal trochophores were eaten more often by all predators (except *A. victoria,* which did not eat eggs). The methods by which setae function defensively have not been investigated, but the radial splay of setae could create at least three potential defenses: (1) the effective size of a larva increases; (2) a buffer zone of setae and water around a larva's tissues is formed; (3) the barbed setae become oriented so that they may pierce objects impinging upon a larva. The possible roles of these mechanisms are discussed below.

Erection of setae increases the overall diameter of a larva, possibly deterring predation by small-mouthed predators as has been shown for freshwater rotifers (Gilbert, 1966). However, the predators used in the present experiments all eat prey much larger than trochophores. Reeve *et al.* (1978) fed *Pseudocalanus minutus* (<650 μm long) to *P. bachei* during production experiments, and Lebour (1924) observed *P. bachei* eating larval fish. *A. victoria* has been commonly observed eating large prey, including fish and other jellyfish (Lebour, 1924; Hyman, 1940; Arai and Jacobs, 1980). The juvenile *Sebastes* sp. fed successfully on *Artemia salina* nauplii (*ca.* 600 μm) as well as upon pieces (>1 mm) of goldfish food. Many species of crab larvae are also cultured successfully on *A. salina* nauplii (Rice and Williamson, 1971) and the megalopa used in these experiments fed on goldfish food as well. It thus seems unlikely that the size increase created by setal erection prevents predation by any of the predator species used here. However, megalopa have far smaller mouths than the other predators tested; erected setae may substantially increase handling difficulty if

megalopa cannot swallow larvae whole but must manipulate and dismember them. Similarly, spines of cyclomorphic cladocerans and rotifers have been shown to reduce predation by freshwater predators with small mouths (reviewed by Zaret, 1980). It thus seems probable that setae function defensively against small-mouthed predators such as megalopa by increasing handling time. In contrast, the other predator species used here could easily swallow whole setose *S. cementarium* trochophores.

The buffer zone of sea water surrounding a trochophore with erected setae may be important as a defense against medusae and tentaculate ctenophores. As described above, both *P. bachei* and *A. victoria* must sense and capture indivdual prey. If a predator's tentacles touch only the erected setae of a trochophore, the tactile or chemical cues necessary to elicit a response may not be perceived. Further, even if a larva is recognized as food, nematocysts and colloblasts may work inefficiently upon setae or across the buffer zone (*ca.* 150 μm) of water created by the setae. If trochophores are first trapped by nematocysts or colloblasts, then ingested and finally expelled, their chances of surviving are probably slim. The numerous trochophores surviving experiments appeared to be in good shape; few were deformed or entangled in mucus. It seems unlikely the surviving trochophores were captured at all by these predators, but that setae prevented recognition or prey capture.

Setae may also deter predation by irritating mouthparts as originally implied by Wilson (1929), whose suggestion seems intuitively reasonable because fish capture prey within the buccal cavity where setae could easily pierce oral tissues as trochophores are bitten or swallowed. Predatory fish are also deterred by the spines of sticklebacks (Hoogland *et al.*, 1957), but the spines of some cyclomorphic rotifers and cladocerans are not considered to be effective against fish (Greene, 1983).

Other work on predation upon marine larvae has found patterns of predation comparable to those presented here. For a predator who senses individual prey at a distance, Landry (1978) found that weakly motile early copepod nauplii were poorly detected by the copepod *Labidocera trispinosa,* and were thus eaten at low rates. Large active nauplii were eaten at the highest rates, while copepodids developed an escape response and were eaten rarely. Also, work with marine fish larvae as prey for various crustaceans has generally found that non-motile eggs are not detected by predators and eaten rarely while motile yolk-sac larvae are eaten at high rates. Feeding larvae develop an escape response and are captured and eaten much less often (Lillielund and Lasker, 1971, Theilaker and Lasker, 1974; Bailey and Yen, 1983). The low rates of predation upon later stages in all cases are due to the development of fundamentally new structures or behaviors during ontogeny, processes not observed for freshwater prey (Greene, 1983).

At present it is not possible to assess the potential impact these predators have on pelagic larval populations of *S. cementarium.* No quantitative estimates of the densities of any of the predator or prey species have been made in the Puget Sound area, though all are common in the plankton during summer. Similarly, except for *P. bachei* (see Reeve and Walter, 1978), quantitative observations of the predation rates of the predators upon other prey types have not been made. However, for the predators used we have shown that rates of predation upon setose trochophores are low.

Susceptibility to predation is a ubiquitous and important problem for embryos and larvae of benthic invertebrates (Thorson, 1946; Young and Chia, in press) which should generate strong selective pressures for larval defense. If effective defenses have evolved, larval forms, behaviors, chemicals, and ultimately reproductive strategies should reflect such selection. Reproduction in many benthic invertebrates with pelagic larvae is characterized by a short period of rapid embryogenesis followed by a prolonged

period of larval feeding and growth. This pattern may be facilitated by the development of efficient larval defenses.

ACKNOWLEDGMENTS

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LITERATURE CITED

ARAI, M. N., AND J. R. JACOBS. 1980. Interspecific predation of common Strait of Georgia planktonic coelenterates: laboratory evidence. *Can. J. Fish. Aquat. Sci.* **37:** 120–123.

BAILEY, K. M., AND J. YEN. 1983. Predation by a carnivorous marine copepod, *Euchaeta elongata* Esterly, on eggs and larvae of the Pacific Hake, *Merluccius productus. J. Plankton Res.* **5:** 71–82.

BHAUD, M., AND C. CAZAUX. 1982. Les larves de polychetes des cotes de France. *Oceanis* **8:** 57–160.

BLAKE, J. A. 1969. Reproduction and larval development of *Polydora* from northern New England (Polychaeta: Spionidae). *Ophelia* **7:** 1–63.

CHRISTIANSEN, F. B., AND T. M. FENCHEL. 1979. Evolution of marine invertebrate reproductive patterns. *Theor. Pop. Biol.* **16:** 267–282.

COWDEN, C., C. M. YOUNG, AND F. S. CHIA. 1984. Differential predation on marine invertebrate larvae by two benthic predators. *Mar. Ecol. Prog. Ser.* **14:** 145–149.

DODSON, S. I. 1974. Adaptive change in plankton morphology: a new hypothesis of cyclomorphosis. *Limnol. Oceanogr.* **19:** 721–729.

FAUCHALD, K. 1974. Polychaete phylogeny: a problem in protostome evolution. *Syst. Zool.* **23:** 493–506.

FRANC, J-M. 1978. Organization and function of ctenophore colloblasts: an ultrastructural study. *Biol. Bull.* **155:** 527–541.

GERRITSEN, J., AND J. R. STRICKLER. 1977. Encounter probabilities and community structure in zooplankton: a mathematical model. *J. Fish. Res. Bd. Can.* **34:** 72–82.

GILBERT, J. J. 1966. Rotifer ecology and embryological induction. *Science* **151:** 1234–1237.

GREENE, C. H. 1983. Selective predation in freshwater zooplankton communities. *Int. Rev. Ges. Hydrobiol.* **68:** 297–315.

HARDY, A. 1956. *The Open Sea: Its Natural History.* Houghton Mifflin Co., Boston. 322 pp.

HOOGLAND, R., D. MORRIS, AND N. TINBERGEN. 1957. The spines of sticklebacks (*Gasterosteus* and *Pygosteus*) as means of defense against predators (*Perca* and *Esox*). *Behavior* **10:** 205–236.

HYATT, K. D. 1979. Feeding strategy. *Fish Physiol.* **8:** 71–120.

HYMAN, L. H. 1940. Observations and experiments on the physiology of medusae. *Biol. Bull.* **79:** 282–296.

JACKSON, G. A., AND R. R. STRATHMANN. 1981. Larval mortality from offshore mixing as a link between precompetent and competent periods of development. *Am. Nat.* **116:** 16–26.

KERFOOT, W. C. 1975. The divergence of adjacent populations. *Ecology* **56:** 1298–1313.

KERFOOT, W. C. 1978. Combat between predatory copepods and their prey: *Cyclops, Epischura, Bosmina. Limnol. Oceanogr.* **23:** 1089–1102.

KERFOOT, W. C. 1980. Perspectives in cyclomorphosis. Pp. 470–476 in *Evolution and Ecology of Zooplankton Communities,* W. C. Kerfoot, ed. University Press of New England, Hanover.

KISLALIOGLU, M., AND R. N. GIBSON. 1976. Some factors governing prey selection by the 15-spined stickleback, *Spinachia spinachia* (L.). *J. Exp. Mar. Biol. Ecol.* **25:** 159–169.

LANDRY, M. R. 1978. Predatory feeding behavior of a marine copepod, *Labidocera trispinosa. Limnol. Oceanogr.* **23:** 1103–1113.

LEBOUR, M. V. 1919. The feeding habits of some young fish. *J. Mar. Biol. Assoc. U.K.* **12:** 9–21.

LEBOUR, M. V. 1924. The food of plankton organisms, II. *J. Mar. Biol. Assoc. U.K.* **13:** 70–92.

LILLIELUND, K., AND R. LASKER. 1971. Laboratory studies of predation by marine copepods on fish larvae. *Fish. Bull.* **69:** 655–667.

LONG, J. A. 1964. The embryology of three species representing three superfamilies of articulate Brachiopoda. Ph.D. Thesis, University of Washington.

MARSHALL, S. M., AND A. P. ORR. 1960. Feeding and nutrition. Pp. 227–258 in *The Physiology of Crustacea, Volume I, Metabolism and Growth,* T. H. Waterman ed. Academic Press, New York and London.

McLAUGHLIN, P. A. 1982. Comparative morphology of crustacean appendages. Pp. 197–256 in *The Biology of Crustacea, Volume II, Embryology, Morphology and Genetics,* L. G. Abele ed. Academic Press, New York and London.

PANTIN, C. F. A. 1942. The excitation of nematocysts. *J. Exp. Biol.* **19:** 294–310.

PECHENIK, J. A. 1979. Role of encapsulation in invertebrate life-histories. *Am. Nat.* **114:** 859–870.

REEVE, M. R., AND M. A. WALTER. 1978. Nutritional ecology of ctenophores—a review of recent research. *Adv. Mar. Biol.* **15:** 249–287.

REEVE, M. R., M. A. WALTER, AND T. IKEDA. 1978. Laboratory studies of ingestion and food-utilization in lobate and tentaculate ctenophores. *Limnol. Oceanogr.* **23:** 740–751.

RICE, A. L., AND D. I. WILLIAMSON. 1971. Methods for rearing larval decapod Crustacea. *Helgol. Wiss. Meersunters.* **20:** 417–434.

SCHROEDER, P. C., AND C. O. HERMANS. 1975. Annelida: Polychaeta. Pp. 1–214 in *Reproduction of Marine Invertebrates,* Volume III, A. C. Giese and J. S. Pearse, eds. Academic Press, New York and London.

SMITH, P. R. 1981. Larval development and metamorphosis of *Sabellaria cementarium* Moore (Polychaeta: Sabellariidae). Master's Thesis, University of Alberta.

THEILACKER, G., AND R. LASKER. 1974. Laboratory studies of predation by euphausid shrimps on fish larvae. Pp. 287–299 in *The Early Life History of Fish,* J. H. S. Blaxter ed. Springer-Verlag, Berlin.

THORSON, G. 1946. Reproduction and larval ecology of Danish marine bottom invertebrates. *Biol. Rev.* **25:** 1–45.

VANCE, R. R. 1973. On reproductive strategies in marine benthic invertebrates. *Am. Nat.* **107:** 339–352.

WILSON, D. P. 1929. The larvae of the British sabellarians. *J. Mar. Biol. Assoc. U.K.* **16:** 221–269.

WILSON, D. P. 1932. On the mitraria larva of *Owenia fusiformis* della Chiaje. *Phil. Trans. R. Soc. Lond.* **B221:** 231–334.

YOUNG, C. M., AND F. S. CHIA. (In press). Abundance and distribution of pelagic larvae as influenced by predatory, behavioral and hydrographic factors. In *Reproduction of Marine Invertebrates,* A. C. Giese and J. S. Pearse, eds. Academic Press, New York and London.

ZAR, J. H. 1974. *Biostatistical Analysis.* Prentice-Hall, Inc., New Jersey. 620 pp.

ZARET, T. M. 1980. *Predation and Freshwater Communities.* Yale Univ. Press, New Haven. 187 pp.

Reference: *Biol Bull.* **167**: 176–185. (August, 1984)

SUN AND SHADE MEDIATE COMPETITION IN THE BARNACLES *CHTHAMALUS* AND *SEMIBALANUS*: A FIELD EXPERIMENT

DAVID S. WETHEY

Department of Biology and Marine Science Program, University of South Carolina, Columbia, South Carolina, 29208

ABSTRACT

The barnacles *Chthamalus fragilis* and *Semibalanus balanoides* compete for space in the high intertidal zone in southern New England. *Chthamalus* settles throughout the intertidal and persists in the absence of competition with *Semibalanus*. *Semibalanus* also settles throughout the intertidal but is usually eliminated from the high intertidal zone by heat and/or desiccation. In a field experiment in the high intertidal zone, *Semibalanus* survived the high summer temperatures and overgrew *Chthamalus* under an opaque roof. Under a transparent roof and in control areas with no roof, *Semibalanus* died in mid summer, and *Chthamalus* persisted. Hence the intensity of interspecific competition is mediated by physical stress which primarily affects the dominant competitor.

INTRODUCTION

A paradigm of intertidal zonation is that local upper shore limits are set by physical stress (heat, desiccation) and local lower shore limits are set by biotic interactions (predation, competition) (*e.g.*, Connell, 1961, 1972, but see also Underwood and Denley, 1984). A corollary is that the intensity of interspecific interactions should decrease as one approaches a local upper shore limit, because the lower shore species should increasingly suffer from physical stress. A second corollary is that in the absence of the physical stress, a low shore species should be able to exclude a high shore species on the high shore. There are several possible tests of these hypotheses. Connell (1961) transplanted a high shore species below its lower shore limit and showed that it survived in the absence of competitors, but died in the presence of competitors. The reverse experiment, reducing the physical stress and determining the outcome of competition, has not been done.

An earlier paper (Wethey, 1983) gave evidence from field transects that the outcome of competition between the barnacles *Semibalanus* and *Chthamalus* was mediated by heat and/or desiccation stress acting primarily on *Semibalanus*. On transects in Connecticut, *Semibalanus* was found at higher levels on the shore in shaded locations than in sunny areas. The upper shore limit of *Semibalanus* distribution was higher in damp areas than in adjacent dry locations (Wethey, 1983). *Chthamalus* was abundant in areas where *Semibalanus* was absent (Wethey, 1983). In low shore areas from which I removed *Semibalanus*, *Chthamalus* survived for over a year well below its normal lower intertidal distribution limit (Wethey, 1983 and unpub.).

There is one field experiment that implicated direct sun (rather than emersion) as a limiting factor on *Semibalanus*. Hatton (1938, p. 274) fixed a sun shade several centimeters above a south facing rock surface and noted after 13 months that

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Semibalanus in the experimental shade area had grown bigger than those in direct sun. The growth rates on shaded south-facing sites were indistinguishable from those on north-facing surfaces that were naturally shaded. The effects of heating by the sun were probably more important than drying of the surface, because the experimentally shaded sites dried out at low tide whereas the north facing rocks did not (Hatton, 1938, p. 275). This experiment was unreplicated and had no controls for the effects of the structure of the shade. Hatton did not report on the effect of the experiment on competition between *Semibalanus* and *Chthamalus*.

In this paper I provide experimental evidence for the influence of physical stress on the intensity of competition between two rocky intertidal barnacles. I show that, as I had previously suggested (Wethey, 1983), the intensity of competition is determined on the high shore by physical stress, which primarily affects the dominant competitor.

Materials and Methods

The study was carried out at the Yale University Peabody Museum Field Station, Guilford, Connecticut. Field experiments were established on the south shore of Horse Island, Long Island Sound (41°16′N, 72°45′W). The smooth granite shore has a 15° slope to the southwest. The tidal range is approximately 1.9 meters, and experiments were established at +1.5 m.

Seven treatments were used to test the effects of shade on the distribution and abundance of *Chthamalus* and *Semibalanus*. The treatments were:

1. Unmanipulated site.
2. Two-sided cage (roofless except for mesh, upper and lower shore sides open).
3. Full cage (roofless except for mesh).
4. Two-sided cage with clear plastic roof (upper and lower shore sides open).
5. Full cage with clear plastic roof.
6. Two-sided cage with opaque plastic roof (upper and lower shore sides open).
7. Full cage with opaque plastic roof.

Galvanized steel hardware cloth cages (10 cm l × 10 cm w × 3 cm h, 1.5 cm mesh) were attached with stainless steel screws set in plastic wall anchors in holes drilled in the rock. Roofs of clear Plexiglas were held onto the tops of the cages with the attachment screws. In the shade treatments, the roofs were wrapped with duct tape, making them opaque. Clear roof treatments, roofless treatments, and unmanipulated areas were used as controls for testing the effects of shade and the effects of the presence of a roof. The design was also used to test for the effects of a cage and cage structure. Three replicates of each treatment were established.

Photographs of each of the sites were taken on 14 June 1983 at initiation of the experiment and on 17 July, 10 August, 19 October, and 1 December, 1983. A focal framer on a 3:1 closeup ring provided registration of camera position (Nikonos, 35 mm lens, flash-lit, Panatomic-X film). The number of individuals of each species in each site was counted on enlargements of the photographs. Percent cover was estimated by placing a transparent sheet with 49 uniformly spaced dots over the enlargements. The percent of the dots touching *Semibalanus*, or *Chthamalus* is an estimate of the percent cover of each species (*e.g.*, Menge, 1976; Wethey, 1983). All *Semibalanus* were counted in the percent cover and census measures. Only the *Chthamalus* that were present at the beginning of the experiment were counted. *Semibalanus* settlement had finished before the start of the experiment, but *Chthamalus* larvae settled during the period August to October.

The tests for the effects of shade, and the various controls were made as follows:

1. The control for the presence of an opaque roof is a clear roof. The effects of a roof in the absence of shade were determined by comparing the roofless treatments to the clear roof treatments.
2. The effects of shade in the presence of a roof were tested by comparing the clear roof treatments to the opaque roof treatments.
3. The the control for the presence of a cage is a two-sided cage. The effect of a cage was tested by comparison of the roofless full cages to roofless two-sided cages.
4. The control for the presence of the support structure (wire mesh) was the unmanipulated area. The comparison of unmanipulated areas with two-sided cage treatments is a test of the effect of the presence of the structure.

These tests were all pre-planned contrasts, which were carried out as part of an analysis of variance. Two parameters were tested: change in percent cover of both *Semibalanus* and *Chthamalus*, and percent survival of the two species. Data were transformed by the arcsin transformation prior to analysis to normalize the distributions.

Because all shade and roof treatments had both full cage and two-sided cage supports, it was necessary to test for the effect of the cage before proceeding with the rest of the analysis. If the cage effect is not significant then the remaining pre-planned contrasts can be used. Despite the fact that sums of squares for caged and two-sided treatments are pooled in the shade and roof contrasts, the tests are considered *a priori* because they were all planned in advance (were not suggested by the data), and only a specific limited subset of all possible comparisons was made. The confidence level (*P*-value) only applies to each particular test, not to the whole series of tests, and is only appropriate when the test is pre-planned (*e.g.*, Neter and Wasserman, 1974, p. 472).

If the only important effects are those of sun *versus* shade, then only the shade test should be significant.

Percent cover data were used only to calculate the change in occupation of space between initiation and termination of the experiment. The uniformly spaced dots tended to fall on the same locations on the two samples, making the estimate of absolute change in percent cover more accurate than would be possible with truly spatially independent samples. Because only a difference in percent cover is calculated, the lack of independence does not compromise the analysis. The test of the effect of treatments on changes in percent cover is equivalent to a test of the effect of treatments on survival of a cohort of individuals.

Results

All sites were equivalent in terms of percent cover at the initiation of the experiment (Table I). This means that any differences detected at the end of the experiment are the results of the treatments, not historical effects carried through from initiation.

Population densities of *Semibalanus* were 10.9 (S.D. 3.4) individuals/cm^2 at initiation of the experiment, which was approximately 60 days after settlement (Table I). Densities at settlement were likely to have been much higher than this, since mortality is high in the period soon after settlement (*e.g.*, Connell, 1961; Wethey, 1984).

There were no effects of caging on the survival of *Chthamalus* or *Semibalanus* (Tables II, III). The tests for effect of the structure of the support (two-sided cages *versus* unmanipulated sites) indicated no structure effect (Tables II, III). The experiments were set up at a tidal level above the local upper foraging limit of the primary

TABLE I

Percent cover of Semibalanus *and* Chthamalus *at initiation on 14 June, 1983*

Semibalanus

Treatment	Mean	SE	
Roofless	57	5	
Clear Roof	62	5	
Opaque Roof	57	5	
Unmanip	58	7	
Contrast	**DF**	**F**	***P***
Roof	1	1.77	0.2047
Shade	1	0.47	0.5042
Cage	1	0.99	0.3356
Structure	1	1.10	0.3111
MSE = 1490, df = 14			

Chthamalus

Treatment	Mean	SE	
Roofless	22	5	
Clear Roof	15	5	
Opaque Roof	20	5	
Unmanip	14	7	
Contrast	**DF**	**F**	***P***
Roof	1	0.50	0.4927
Shade	1	0.19	0.6723
Cage	1	0.65	0.4352
Structure	1	0.09	0.7685
MSE = 310, df = 14			

Analysis of variance carried out on arcsin-square root transformed percentages. The mean values are transformed back to percents. Abbreviations: DF = degrees of freedom, MSE = mean square error, F = variance ratio, *P* = probability of Type I error. Roof contrast is *a priori* comparison of roofless and clear roof treatments. Shade contrast is *a priori* comparison of clear roof and opaque roof treatments. Cage contrast is *a priori* comparison of roofless cages to roofless two-sided cage treatments. Structure contrast is *a priori* comparison of roofless two-sided cages to unmanipulated treatments. Population densities at initiation: *Semibalanus* 10.9/cm^2 (S.D. 3.43), *Chthamalus* 1.3/cm^2 (S.D. 1.06).

predator (*Urosalpinx cinerea*). The cages served primarily to exclude the herbivorous gastropod *Littorina littorea* (mean number per quadrat in July, August, and October samples: cage 0.3, two side 2.1). *Littorina saxatilis* were small enough to enter the cages and were found in abundance at each survey (for example, mean number per quadrat in July samples; cage 27.9, two side 26.9). The primary effect of the roofless and clear roof cages was the growth of a canopy of green filamentous algae from June to October (canopy appeared in 3 of the 6 unshaded cages, percent cover in August 44%, S.D. 33%), and the appearance of a few *Fucus vesiculosus* plants thereafter (*Fucus* appeared in 2 roofless cages, 4 plants in one and 1 plant in the other). The canopy of filamentous green algae evidently did not provide enough shade or water retention to mitigate the effects of sun on *Semibalanus*. Because there was no cage effect on survival of *Semibalanus* or *Chthamalus*, the pre-planned pooling of data from full cages and two sided cages was retained from the tests of the shade and roof effects.

TABLE II

Percent survival of Semibalanus *from June to December*

Treatment	Mean	SE	
Roofless	7	3	
Clear Roof	7	3	
Opaque Roof	21	3	
Unmanip	0	4	
Contrast	**DF**	**F**	***P***
Roof	1	0.01	0.906
Shade	1	7.64	0.015
Cage	1	0.21	0.657
Structure	1	1.11	0.309
MSE = 305, df = 14			

Analysis of variance carried out on arcsin-square roof transformed percentages. The mean values are transformed back to percents. Abbreviations and contrasts are as in Table I.

Percent survival of *Semibalanus* was higher in the shade treatments than in the treatments exposed to direct sun (roofless and clear roof) (Table II). Survival was significantly less under the clear roof (7%) than under the opaque roof (21%) (shade effect, Table II). There was no effect of the presence of the roof alone, based on comparison of the clear roof (7% survival) and roofless (7% survival) treatments (roof effect, Table II). Therefore the shade effect is the result of the shade alone and is not confounded by the presence of the structure of the roof.

Percent survival of *Chthamalus* was lower in the shade treatments than in the treatments exposed to direct sun (Table III). Survival under the clear roof (76%) was significantly higher than under the opaque roof (36%) (shade effect, Table III). There was no effect of the presence of the roof alone, based on comparison of the clear roof (76% survival) and roofless (67% survival) treatments (roof effect, Table III). Therefore the shade effect is the result of the shade alone, and is not confounded by the presence of the roof.

TABLE III

Percent survival of Chthamalus *from June to December*

Treatment	Mean	SE	
Roofless	67	12	
Clear Roof	76	12	
Opaque Roof	36	12	
Unmanip	95	17	
Contrast	**DF**	**F**	***P***
Roof	1	0.32	0.5805
Shade	1	5.64	0.0324
Cage	1	0.03	0.8611
Structure	1	2.62	0.1278
MSE = 1330, df = 14			

Analysis of variance carried out on arcsin-square root transformed percentages. The mean values are transformed back to percents. Abbreviations and contrasts are as in Table I.

Occupation of space was dramatically affected by the experimental treatments. In the shade treatments *Semibalanus* increased its occupation of space (Table IV) and formed 2.5 cm tall hummocks under the roofs. In the clear roof and roofless treatments, occupation of space by *Semibalanus* decreased (Table IV); any surviving *Semibalanus* were relatively small and no hummocks formed. *Chthamalus* slightly decreased (not statistically significantly) in occupation of space in the shade treatments and remained constant in the clear roof and roofless treatments (Table IV).

The majority of the deaths of *Chthamalus* in the shade treatments were the result of direct interactions with *Semibalanus*. *Chthamalus* individuals were overgrown, crushed, and undercut by *Semibalanus* (Table V). Individuals that were far enough away from *Semibalanus* that they did not experience direct interference, survived in the shade treatments. Few *Chthamalus* individuals in the sun treatments (roofless and clear roof) were close enough to surviving *Semibalanus* to sustain damage. The *Chthamalus* that were close to *Semibalanus* died as a result of the interaction. There were proportionally very few deaths of *Chthamalus* as a result of unknown causes or of interactions with conspecifics (Table V).

TABLE IV

Changes in space occupation from June to December

Semibalanus

Treatment	Mean	SE	
Roofless	−34.7	15.4	
Clear Roof	−46.4	15.4	
Opaque Roof	30.4	15.4	
Unmanip	−86.4	21.7	
Contrast	**DF**	**F**	***P***
Roof	1	0.08	0.7833
Shade	1	11.38	0.0046
Cage	1	0.35	0.5618
Structure	1	1.21	0.2908
MSE = 2215, df = 14			

Chthamalus

Treatment	Mean	SE	
Roofless	3.4	10.4	
Clear Roof	0.01	10.4	
Opaque Roof	−25.2	10.4	
Unmanip	26.1	14.7	
Contrast	**DF**	**F**	***P***
Roof	1	0.10	0.7623
Shade	1	2.12	0.1674
Cage	1	0.22	0.6468
Structure	1	0.46	0.5075
MSE = 2689, df = 14			

Analysis of variance carried out on arcsine-square root transformed percentages. Mean values transformed back to percents. Values are changes in percent cover from the beginning to the end of the study (positive values indicate an increase in percent cover, negative values indicate a decrease). Abbreviations and contrasts are as in Table I.

TABLE V

Causes of death of Chthamalus

Cause	Shade trtmts. percent	Sun trtmts. percent
Killed by *Semibalanus*		
Overgrown	73	6
Crushed	13	34
Undercut	7	21
Killed by *Semibalanus* and *Chthamalus*		
Crushed	0	2
Killed by *Chthamalus*		
Crushed	0	4
Unknown	6	34

Values are percents of total deaths from particular identifiable causes in the shade and sun treatments. Shade treatments = opaque roof. Sun treatments = clear roof and roofless. Shade treatments: number of individuals = 130, sun treatments: number of individuals = 53.

DISCUSSION

This study examined the influence of physical factors (heat and/or desiccation) on the intensity of interspecific competition between the barnacles *Semibalanus* and *Chthamalus* near the northern geographic limit of *Chthamalus* in New England. In sunny locations the upper shore limit of *Semibalanus* distribution is lower than in more shaded locations and *Chthamalus* survives in areas where *Semibalanus* dies (Wethey, 1983). I argued that the intensity of the competitive interactions between *Chthamalus* and *Semibalanus* were mediated by intolerance of heat and/or desiccation by *Semibalanus* (Wethey, 1983). The experiment described here is a field test of this hypothesis.

The results of the experiment are consistent with the shade/competition hypothesis. In the shade treatments *Semibalanus* survived (Table II) and grew to form hummocks 2.5 cm high. It increased occupation of space at the expense of *Chthamalus* (Tables III, IV, V). In the clear roof and the roofless treatments *Semibalanus* died (Table II) and its occupation of space decreased during the experiment (Table IV). *Chthamalus* remained unchanged in the sun treatments (Tables II, III, IV). No hummocks formed in any of the sun treatments. The results were striking enough that at termination the shade treatments were recognizable from a distance of several meters away on the shore after the hardware was removed.

The controls for the effect of the roof alone and the support structure alone are essential to allow the results of the experiment to be applied to the real world. These controls allow one to separate the effect of shade from the effect of the structure holding the shade above the experimental plots. The clear roof controls were indistinguishable from the roofless treatments, indicating that shade alone was the important factor affecting survival in the shade treatments (Table II). Therefore the results of the experiment can be applied to any locations where shade occurs naturally. The survival of *Semibalanus* is therefore strongly influenced by shade in southern New England.

These results are consistent with the hypothesis that the upper shore limit of *Semibalanus* is set by intolerance of heat and/or desiccation rather than intolerance of emersion. The effect of the shade treatment was similar to that reported by Hatton (1938). He concluded that shade alone strongly influenced growth. Other experiments could not distinguish between the effect of emersion and the effect of heat/desiccation. Hatton (1938) reduced the importance of desiccation and raised the upper shore limit of *Semibalanus* by means of the drips from a slowly draining basin fixed in the high intertidal. Hatton's (1938) drip treatment also may have added food to the experimental individuals (Underwood and Denley, 1984). Foster (1969, 1971a, b) concluded that heat and desiccation were important, based on field and laboratory experiments and observations of changes in the upper shore limit of *Semibalanus* in mid summer (see also Bowman, 1982). Connell (1961b) found that mortality rates measured during hot dry weather were greater than those measured in cooler periods and concluded that desiccation was important. In northern Scotland, *Chthamalus* is more abundant on surfaces that dry out at low tide and *Semibalanus* is more abundant on surfaces that remain wet (Lewis, 1964). *Semibalanus* is more common on north-facing than south-facing shores in the south of England (Crisp and Southward, 1958). As one approaches the southern limit of *Semibalanus,* it become progressively more restricted to shaded locations (Barnes, 1958). The present study provides the first controlled experimental demonstration that shade alone can determine the local upper shore limit.

Underwood and Denley (1984) state that one cannot make the generalization that local upper limits are set by physical stress operating after larval settlement. They erect several alternative hypotheses: animals on the high shore may starve during calm weather because they are not submerged long enough to feed. Alternatively larvae may actively avoid settlement on the high shore. In addition transplant ". . . experiments only reveal sources of mortality of organisms moved outside their normal zone and do not tell us anything definite about reasons for the absence of organisms from such areas" (Underwood and Denley, 1984). The present experiments falsify all of these alternatives posed by Underwood and Denley (1984) and demonstrate that physical stress operating after larval settlement directly limits the upper shore distribution limit of *Semibalanus* in southern New England.

On a local scale the distribution of sun and shade will likely correlate strongly with the distribution of *Semibalanus* and *Chthamalus*, with the latter being prevalent in sunny sites on the high shore (*e.g.,* Wethey, 1983). *Semibalanus* is the dominant competitor and exerts a strong influence on the distribution and abundance of *Chthamalus.* In the treatments where *Semibalanus* survived, it killed neighboring *Chthamalus* individuals by overgrowing, crushing, or undercutting them (Table V). In the absence of *Semibalanus, Chthamalus* survived (Tables II, III, V). Hence the survival of *Semibalanus* to a large extent determines the fate of *Chthamalus* as a result of competition for space. The intensity of competition is in turn determined by the action of heat and desiccation on *Semibalanus.* In sunny sites on the high shore, *Semibalanus* dies and *Chthamalus* experiences little competition (Tables II, III, V). In shaded sites *Semibalanus* survives and *Chthamalus* loses in competition (Tables II, III, V). Thus local zonation is likely the result of postsettlement mortality from interspecific competition in *Chthamalus* rather than the result of any requirements for dry conditions on the part of *Chthamalus* adults or juveniles. These results are consistent with those of Connell (1961) who showed that interspecific competition with *Semibalanus* had a much greater influence on the distribution of *Chthamalus* than did intraspecific competition, or larval settlement pattern.

On a geographic scale, *Chthamalus* is likely to persist only in areas where *Semibalanus* predictably dies on the high shore, providing a refuge from competition (Wethey, 1983). In Massachusetts north of Cape Cod, *Semibalanus* does not die on the high shore (Wethey, 1983), and as a result its upper shore limit does not change during the year. At this location, *Semibalanus* does not settle above the upper shore limit of adults. At Nahant, Massachusetts (north of Cape Cod), I have monitored settlement in sites 10 cm above the upper shore limit of *Semibalanus* and have not seen more than 1 cyprid larva/cm^2 (unpub.). This is in striking contrast to the pattern in Connecticut, where I measured densities of *Semibalanus* metamorphosed spat of 10/cm^2 in June (6 weeks after the end of settlement) at shore levels 20 cm above the upper shore limit of adult distribution (Table I). *Chthamalus* is absent at Nahant and is abundant on the high shore in Connecticut.

On a temporal scale, after a period of hot summers, *Chthamalus* would be expected to increase in abundance, as a result of the lessening of the intensity of competition with *Semibalanus.* After a period of cold summers, *Chthamalus* should decrease as a result of the greater intensity of competition with *Semibalanus.* Such a temporal pattern has been documented by Southward and Crisp (1956), Southward (1967) and Crisp *et al.* (1981) in England. The abundance of *Chthamalus* in southern New England may have increased since the climatic minimum in the early 1800's. The species was noted by Darwin (1854) in collections from Charleston, South Carolina, but not in collections from Delaware Bay or Massachusetts. It was first noted at Woods Hole in 1898 by M. A. Bigelow (Sumner *et al.,* 1913, pp. 191, 646). The first published report was by Sumner (1909). Sumner *et al.* (1913) note "It is hard to believe that this species has been habitually confused with [*Semi*]*balanus balanoides* by the long succession of field naturalists and systematic zoologists who have exploited the shores of New England for over a century. These men erred rather in the direction of discovering too many new species than in ignoring well established ones." Pilsbry (1916) in his monograph on the North American barnacles was equally puzzled by this. Perhaps *Chthamalus* really was rare in the mid 1800's and reinvaded from the south as a result of release from competition with *Semibalanus* brought about by the climatic warming.

The physical environment and biotic interactions combine to determine the dynamics of this high intertidal barnacle assemblage. The intensity of interspecific competition is mediated by physical stress, which primarily affects the dominant competitor. *Semibalanus* can significantly reduce the population densities of *Chthamalus,* even on the high shore, if *Semibalanus* is not killed by physical stress. An understanding of the interplay between physical stress and biotic interactions may allow us to understand not only local zonation, but also geographic limits of species and patterns of long term temporal variation in relation to climatic change.

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LITERATURE CITED

BARNES, H. 1958. Regarding the southern limits of *Balanus balanoides* (L.). *Oikos* **9:** 139–157.

BOWMAN, R. S. 1982. The role of stochastic events in *Balanus/Chthamalus* interactions on Scottish shores. *Abstr. 17th Eur. Mar. Biol. Symp.*

CONNELL, J. H. 1961. The influence of interspecific competition and other factors on the distribution of the barnacle *Chthamalus stellatus*. *Ecology* **42:** 710–723.

CONNELL, J. H. 1972. Community interactions on marine rocky intertidal shores. *Ann. Rev. Ecol. Syst.* **3:** 169–172.

CRISP, D. J., AND A. J. SOUTHWARD. 1958. The distribution of intertidal organisms along the coasts of the English Channel. *J. Mar. Biol. Assoc. U. K.* **37:** 157–208.

CRISP, D. J., A. J. SOUTHWARD, AND E. C. SOUTHWARD. 1981. On the distribution of the intertidal barnacles *Chthamalus stellatus, Chthamalus montagui* and *Euraphia depressa*. *J. Mar. Biol. Assoc. U. K.* **61:** 359–380.

DARWIN, C. 1854. *A Monograph on the Sub-class Cirripedia.* Ray Society, London. 684 pp.

FOSTER, B. A. 1969. Tolerance of high temperature by some intertidal barnacles. *Mar. Biol.* **4:** 326–332.

FOSTER, B. A. 1971a. Desiccation as a factor in the intertidal zonation of barnacles. *Mar. Biol.* **8:** 12–29.

FOSTER, B. A. 1971b. On the determinants of the upper limit of intertidal distribution of barnacles (Crustacea: Cirripedia). *J. Anim. Ecol.* **40:** 33–48.

HATTON, H. 1938. Essais de bionomie explicative sur quelques especes intercotidales d'algues et d'animaux. *Ann. Inst. Oceanogr.* **17:** 241–348.

LEWIS, J. R. 1964. *The Ecology of Rocky Shores.* Hodder and Stoughton, London. 323 pp.

MENGE, B. A. 1976. Organization of the New England rocky intertidal community: role of predation, competition and environmental heterogeneity. *Ecol. Monogr.* **46:** 355–393.

NETER, J., AND W. WASSERMAN. 1974. *Applied Linear Statistical Models.* Irwin, Homewood Illinois. 842 pp.

PILSBRY, H. A. 1916. The sessile barnacles (Cirripedia) contained in the collections of the U. S. National Museum; including a monograph of the American species. *Bull. U. S. Nat. Mus.* **93:** 1–366.

SOUTHWARD, A. J. 1967. Recent changes in the abundance of intertidal barnacles in south west England: a possible effect of climatic deterioration. *J. Mar. Biol. Assoc. U. K.* **47:** 81–95.

SOUTHWARD, A. J., AND D. J. CRISP. 1956. Fluctuations in the distribution and abundance of intertidal barnacles. *J. Mar. Biol. Assoc. U. K.* **35:** 211–229.

SUMNER, F. B. 1909. On the occurrence of the littoral barnacle *Chthamalus stellatus* (Poli) at Woods Hole, Mass. *Science* **30:** 373–374.

SUMNER, F. B., R. C. OSBURN, AND L. J. COLE. 1913. A biological survey of the waters of Woods Hole and vicinity. *Bull. U. S. Bureau Fisheries* **31:** 1–794.

UNDERWOOD, A. J., AND E. J. DENLEY. 1984. Paradigms, explanations and generalizations in models for the structure of intertidal communities on rocky shores. Pp. 151–180 in *Ecological Communities: Conceptual Issues and the Evidence,* D. R. Strong, Jr., D. Simberloff, L. G. Abele, and A. B. Thistle, eds. Princeton University Press, Princeton.

WETHEY, D. S. 1983. Geographic limits and local zonation: the barnacles *Semibalanus* (*Balanus*) and *Chthamalus* in New England. *Biol. Bull.* **165:** 330–341.

WETHEY, D. S. 1984. Spatial pattern in barnacle settlement: day to day changes during the settlement season. *J. Mar. Biol. Assoc. U. K.* **64:** (in press).

Reference: *Biol Bull.* **167**: 186–199. (August, 1984)

GROWTH RATES OF THE SEA SCALLOP, *PLACOPECTEN MAGELLANICUS*, DETERMINED FROM THE $^{18}O/^{16}O$ RECORD IN SHELL CALCITE

DAVID E. KRANTZ[1], DOUGLAS S. JONES[2], AND DOUGLAS F. WILLIAMS[3]

[1]*Marine Science Program, University of South Carolina, Columbia, South Carolina 29208,* [2]*Department of Geology, University of Florida, Gainesville, Florida 32611, and* [3]*Department of Geology and Belle W. Baruch Institute, University of South Carolina, Columbia, South Carolina 29208*

Abstract

Present age determination techniques for the sea scallop, *Placopecten magellanicus* (Gmelin), rely on the subjective interpretation of lines on the shell exterior as representing periods of annual growth. This study compares scallop age and growth estimates from the external line method with a stable isotope technique. The oxygen isotopic records from serially sampled carbonate powders taken from two scallop specimens collected alive off the Virginia coast show annual cycles which closely approximate the isotopic composition predicted as a function of observed salinity and temperature. Since these annual isotopic cycles are controlled by physical-chemical processes, they provide an independent time scale for age and growth rate determination. Growth rates determined from the isotopic records are roughly twice those estimated from the external line method and from a published average growth curve for *Placopecten magellanicus*.

Introduction

The sea scallop, *Placopecten magellanicus* (Gmelin), is an important economic resource for the New England and Atlantic Canada fisheries, providing the fourth highest income from landings (Bourne, 1964; Serchuk *et al.*, 1979). Average annual production from combined U. S. and Canadian catches is on the order of 13,000 metric tons of meats (Serchuk *et al.*, 1979). Harvestable populations of sea scallops occur from St. Lawrence Bay to the Virginia-North Carolina continental shelf just north of Cape Hatteras (Posgay, 1957; Merrill, 1962). Sea scallops in the northern portion of the range inhabit water depths from approximately one meter below mean low tide to just deeper than 100 meters on the continental shelf (Dickie, 1955). The more southerly populations inhabit increasingly deeper waters. In the Virginia Bight, the southern extreme of their range, sea scallops are confined to 40–100 meter water depths (Merrill, 1971), presumably in response to lethal summer temperatures above 20–23°C (Dickie, 1958) in shallower waters.

The considerable economic importance and heavy fishing of sea scallop stocks underscore the need for accurate age and growth rate estimates for this species. Such information is vital if the sea scallop fishery is to be managed effectively. The techniques which are presently employed for age determination of the sea scallop rely on the interpretation of lines visible on the exterior of the shell or on the hinge ligament (Stevenson and Dickie, 1954; Merrill *et al.*, 1965). Based primarily on mark and recovery studies, several pectinids, including *Placopecten magellanicus*, deposit annual rings or growth lines in late winter or early spring (Stevenson and Dickie, 1954;

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Taylor and Venn, 1978; Serchuk *et al.*, 1979; Paul, 1981). Scallops deposit calcium carbonate in the form of calcite to the shell margin in concentric increments. During the warmer months of the year, while the scallops are growing rapidly, the distance between consecutive increments is relatively wide (Stevenson and Dickie, 1954; Mason, 1957; Taylor and Venn, 1978). As growth slows during the winter and early spring, the growth increments are crowded together, forming what appears to be a concentric line or ring on the shell (Fig. 1).

The interpretation of external lines as representing years of growth for an individual specimen is often complicated by numerous disturbance or shock rings. Sea scallops are notably sensitive to physical disturbances and sudden changes in environmental conditions such as sharp temperature or salinity changes, or storm-related turbulence (Merrill *et al.*, 1965). In response to strong stimuli, scallops retract the mantle and cease calcification along the shell margin. This action leaves a noticeable line after shell growth resumes. Distinguishing annual lines from disturbance lines macroscopically is frequently difficult and often very subjective (Stevenson and Dickie, 1954; Merrill *et al.*, 1965). Other shell characteristics, such as seasonal variation in shell color, convexity of the shell profile, and activity of boring organisms, may provide some information for the interpretation of external lines (Merrill *et al.*, 1965). However, these additional methods are still very subjective and are not conclusive. In view of the uncertainties involved in the accurate interpretation of growth rings, additional methods of establishing the periodicity of these features have been pursued in other species of molluscs. Principal among these efforts has been the investigation of stable isotope variations across shell increments (*e.g.*, Yavnov and Ignat'ev, 1979; Jones *et al.*, 1983).

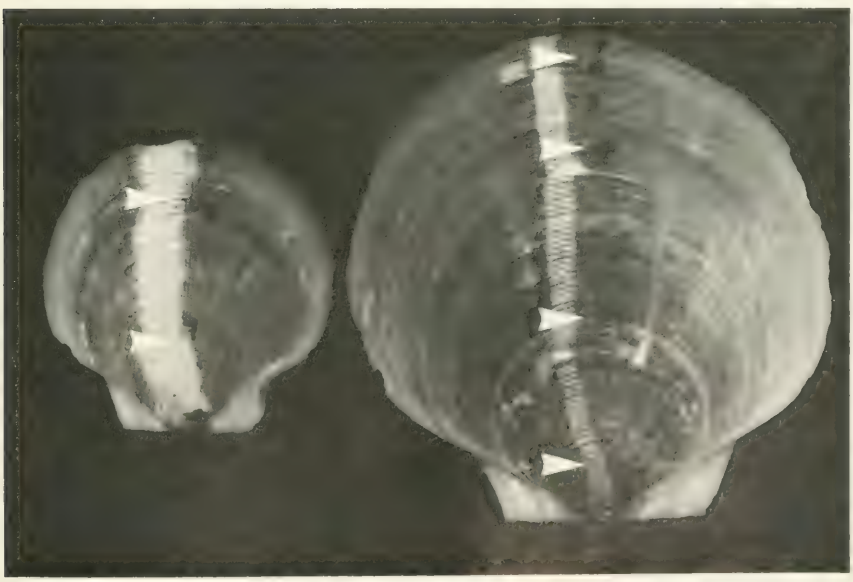

FIGURE 1. Two specimens of *Placopecten magellanicus*, PM10 (left) and PM26 (right), used for isotopic analyses. Years of growth estimated from external lines are marked on the shell with corresponding year number. Position of shell margin for each summer of growth as determined from the isotopic profiles are indicated by white arrows. Grooves drilled to collect carbonate powder samples are visible on the shell exterior.

Application of stable isotopic methods to mollusc studies

This study utilizes the ratio between the stable isotopes of oxygen, ^{18}O and ^{16}O, in scallop shell carbonate to monitor ambient water conditions during shell deposition, and ultimately to make interpretations about shell growth. Because of the thermodynamic behavior of the oxygen isotopes in chemical reactions, the ratio of $^{18}O/^{16}O$ in the product is a function of the $^{18}O/^{16}O$ ratio of the reactants and the temperature at which the reaction occurred (Urey, 1947). During the deposition of shell carbonate by a mollusc, this isotopic fractionation is controlled by the $^{18}O/^{16}O$ ratio of the water in which the animal is living and the ambient temperature during shell deposition. With a constant $^{18}O/^{16}O$ ratio for water, relatively fewer ^{18}O atoms are incorporated into shell carbonate in the warmer summer months ("lighter" isotopic values) and proportionately more ^{18}O atoms are incorporated in the cooler winter months ("heavier" isotopic values). The temperature control on the fractionation of oxygen isotopes is a function of the reaction kinetics and in molluscs is, for the most part, independent of physiological processes (Epstein and Lowenstam, 1953; Epstein *et al.*, 1953; Jones *et al.*, 1983).

The isotopic composition of shell carbonate is also a function of the $^{18}O/^{16}O$ ratio of the water. The water $^{18}O/^{16}O$ is a conservative property and may be related to salinity in that sea water is isotopically heavier (relatively more ^{18}O) than freshwater (Epstein and Mayeda, 1953; Fairbanks, 1982). Shell carbonate deposited by molluscs living in marine conditions will be isotopically heavier than shell carbonate deposited under freshwater conditions (Epstein *et al.*, 1953; Keith *et al.*, 1964; Eisma *et al.*, 1976).

The controlling factors of water $^{18}O/^{16}O$ and temperature have been quantified and related to shell carbonate isotopic composition in the calcite paleotemperature equation (Epstein *et al.*, 1951; Epstein *et al.*, 1953). The principal application of this equation is to calculate temperature of formation from a known carbonate isotopic value when water isotopic composition is known or can be reasonably estimated. Epstein *et al.* (1953) emphasize that seasonal temperature cycles produce significant variations in isotopic composition within the shell. By sequentially sampling a mollusc shell at closely spaced intervals (approximately 1 mm), it is possible to check for seasonal changes in the $^{18}O/^{16}O$ ratio and compare these cycles with the shell growth record. Information obtained by sequential sampling of mollusc shells has allowed the interpretation of annual growth patterns (Wefer and Killingley, 1980; Cochran *et al.*, 1981), periodicity of growth increment formation (Horibe and Oba, 1972; Williams *et al.*, 1982; Jones *et al.*, 1983), and correlation of the shell isotopic record with upwelling events (Killingley and Berger, 1979), seasonal productivity changes, and thermocline development (Arthur *et al.*, 1983).

Hydrographic conditions of the study area

Hydrographic data obtained from 40 to 60 meter water depths in the shelf area near the collection site of specimens PM10 and PM26 were averaged by month for the years 1975 to 1979, which represent the growth period of the specimens. The area of the mid-shelf from which the scallop specimens were collected is essentially full marine with mean salinity of 33.7‰ and an average annual salinity range from 32.7 to 34.7‰ (unpublished data from NOAA-NODC; Nickerson and Mountain, 1983). A certain degree of seasonality is associated with salinity in that highest salinity values occur in the spring and fall, while lowest salinity values occur during mid-summer (Fig. 2). Occasional short-term deviations occur from this average trend, most probably in response to extreme precipitation or local hydrographic fluctuations.

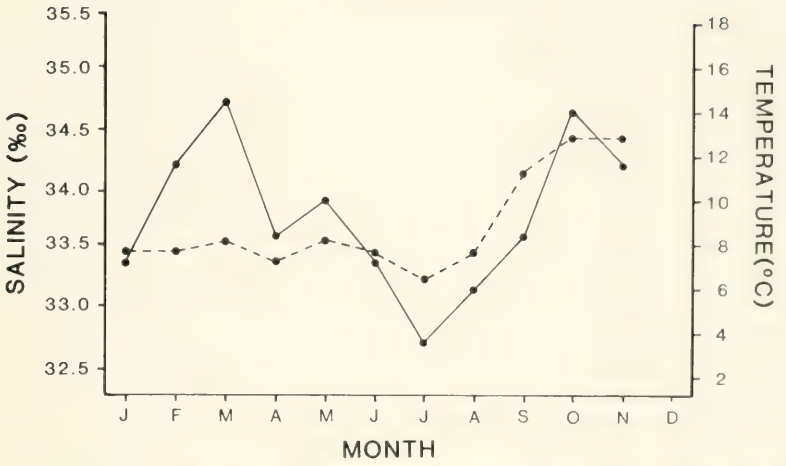

FIGURE 2. Mean monthly bottom water salinity (solid line) and temperature (dashed line) for areas of the Virginia Bight continental shelf with water depths between 40 and 60 meters. Data obtained from NOAA-NODC and Nickerson and Mountain (1983) are summarized for the years 1975–1979.

Average monthly bottom water temperatures for the same area range from approximately 6.0°C to a maximum of approximately 13.0°C. Extreme temperatures may be 2 or 3°C on either side of the average range, dipping to 4°C or rising to 16°C. Throughout most of the winter, spring, and early summer, average bottom water temperatures remain below 8.0°C (Fig. 2). Beginning in late summer and continuing through the fall, bottom water temperatures steadily increase to an annual maximum. An abrupt temperature drop with the onset of winter is followed by a fairly constant low temperature. As in the case of yearly salinity variations, short-term excursions in temperature also occur throughout the year, probably caused by local hydrographic events.

Predicted isotopic composition of shell calcite

The isotopic composition of shell calcite was predicted using: (1) an unpublished regression equation relating the water $\delta^{18}O$ to salinity for the Virginia Bight (R. G. Fairbanks, pers. comm.), (2) the average bottom-water salinity and temperature observations, and (3) the calcite paleotemperature equation (Epstein *et al.*, 1953). The predicted $\delta^{18}O$ of calcite can be estimated by solving the paleotemperature equation (Epstein *et al.*, 1953) using the quadratic formula, such that:

$$\delta^{18}O \text{ (calcite)} = \delta^{18}O \text{ (water)} + [(4.38 - \sqrt{19.18 - 0.4(16.9 - T)})]/0.20$$

where T = temperature in degrees C. Temperature observations and water $\delta^{18}O$ estimates from salinity observations were substituted into this derivation of the paleotemperature equation to predict the average oxygen isotopic composition of shell calcite deposited during each month of the year.

For areas of the Virginia Bight continental shelf with water depths between 40 and 60 meters, the predicted annual isotopic record from scallop shell calcite is as shown in Figure 3. A distinct seasonality is evident with the lightest isotopic values of approximately 0.5‰ occurring in the late summer and early fall, and the heaviest values of approximately 2.5‰ occurring in the winter. Since the curve is based on average hydrographic conditions, any given year of actual conditions may deviate

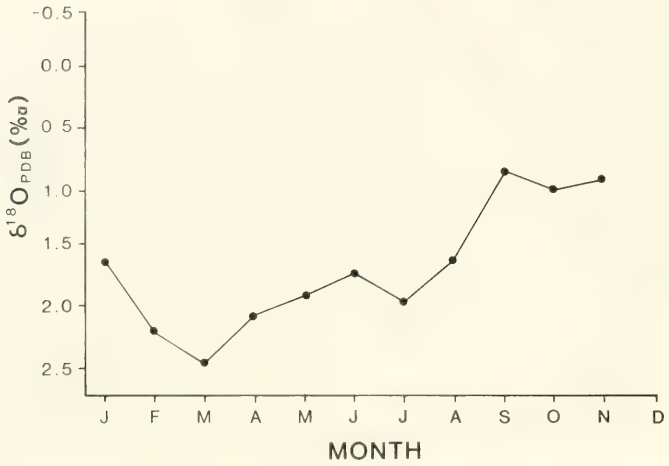

FIGURE 3. Predicted average monthly $\delta^{18}O$ values for scallop shell calcite. Shell carbonate isotopic composition was estimated using average monthly salinity and temperature values (Fig. 2) and the calcite paleotemperature equation (Epstein *et al.*, 1953). A distinct seasonality is evident, with lightest $\delta^{18}O$ values in the late summer and heaviest values in the winter and early spring.

somewhat from this predicted curve, and actual seasonal extremes may be somewhat greater. The short-term temperature and salinity excursions, which are not uncommon on the shelf, would be expected to show up in the shell isotopic record as deviations from the annual trend. However, the seasonal cycle should still be the major signal recorded in the shell carbonate of the scallop, as has been demonstrated for other mollusc species (Wefer and Killingley, 1980; Williams *et al.*, 1982; Jones *et al.*, 1983).

MATERIALS AND METHODS

Living specimens of *Placopecten magellanicus* used in this study were collected by National Marine Fisheries Service (NMFS) personnel during the 1979 yearly shellfish assessment survey. Isotopic analyses were performed on two specimens which were collected alive on 21 May 1979 from a station at 57 meters water depth approximately 90 km off the coast of Virginia (37°15′N, 74°45′W). These two specimens (PM10, PM26) were chosen from a group of thirty because the exterior of both shells showed no evidence of boring or extensive erosion. Estimation of the yearly growth for each individual was made by NMFS personnel using lines on the shell exterior in a method described by Merrill *et al.* (1965). Indelible marks representing estimated years of growth were placed on each shell by NMFS personnel, and were later used for comparison with the growth interpretation based on the stable isotope record.

Both PM10 and PM26 specimens were prepared for isotopic analysis by first lightly grinding the exterior of the shell to remove the periostracum and any foreign debris. Discrete samples of carbonate powder were then drilled from the outer shell layer using a 0.5-mm dental drill bit. The calcium carbonate powders were collected in a series by drilling consecutive grooves parallel to shell growth increments from the umbo to the ventral margin along the axis of maximum growth. Samples were taken only from the outer prismatic shell layer which is deposited sequentially along the shell margin during the growth of the scallop. Care was taken to avoid the inner shell layer which is deposited in thin sheets over existing calcite. Any given point of

the inner shell layer will be composed of calcium carbonate deposited over an extended period of time instead of the single time slice represented by the overlying prismatic layer.

The stable isotopic composition of the shell powders was determined using standardized techniques (Williams *et al.*, 1977; Jones *et al.*, 1983). Approximately 0.5 mg of each carbonate sample was first roasted *in vacuo* at 380°C for one hour to remove any remnant of the organic matrix. Each roasted sample was then reacted in purified phosphoric acid at 50°C using a technique modified from McCrea (1950). The oxygen and carbon isotopic compositions of the evolved carbon dioxide gas were determined on a VG Micromass 602-D mass spectrometer. By convention, the isotopic values are recorded relative to the carbon dioxide gas derived from the Pee Dee Belemnite (PDB) standard carbonate powder (Epstein *et al.*, 1953) in conventional delta notation (δ, ‰). Analytical precision was ±0.10‰ for prepared samples.

Results

Specimen PM10, with a shell height of 75 mm, was estimated by NMFS personnel to have completed three years of growth and begun shell deposition in a fourth year. This age determination was based on an interpretation of the external growth lines represented diagrammatically in Figure 4. The millimeter scale on the horizontal axis relates the position of these external lines to shell height (as measured from the umbo to the margin) and the position of each carbonate powder sample drilled from the shell. Isotopic determinations were made on 46 discrete powders secured from the shell of specimen PM10. The oxygen isotope data (see Krantz, 1983 for data) are plotted in Figure 4 with the $\delta^{18}O$ scale reversed so that lower δ values, representing "warm" isotopic temperatures, are at the top of the vertical scale.

The oxygen isotope record from specimen PM10 exhibits two major cycles with approximately 2‰ variation between minimum and maximum $\delta^{18}O$ values (Fig. 4). The heaviest (most positive) $\delta^{18}O$ value in the record is +2.46‰ and the lightest (most negative) is +0.29‰. The $\delta^{18}O$ curve is roughly sinusoidal although occasional deviations of a few tenths of a per mil are observed from the trend.

As previously discussed, ambient temperatures during shell formation may be estimated from the calcite paleotemperature equation (Epstein *et al.*, 1953) using the $\delta^{18}O$ values of the individual carbonate samples and the $\delta^{18}O$ values of the water. As a first approximation, if one makes the simplifying assumption that changes in water $\delta^{18}O$ at the collection site are negligible, an average water $\delta^{18}O$ value of −0.25‰ (R. G. Fairbanks, pers. comm.) can then be used to estimate temperature during shell deposition. In this manner, the shell carbonate $\delta^{18}O$ values can be converted to temperature minima and maxima. Using this approximation, the minimum isotopic temperature recorded in the shell of PM10 is 6.5°C, the maximum is 14.6°C, and the average range is 7.5°C.

Specimen PM26, with a shell height of 126 mm was collected on the same day and from the same station as specimen PM10. NMFS personnel estimated the age of specimen PM26 to be seven years with the beginning of an eighth year of growth, again relying on the interpretation of external growth lines (Fig. 5). The $\delta^{18}O$ profile of PM26 shows four large amplitude cycles (Fig. 5) with approximately 2‰ variations occurring between minimum and maximum $\delta^{18}O$ values. Superimposed on these cycles are several smaller amplitude excursions of approximately 0.2 to 0.5‰. As with the oxygen isotope data from specimen PM10, the data from PM26 may be converted to isotopic temperature estimates using an average water $\delta^{18}O$ of −0.25‰. The minimum and maximum calculated temperature values vary from 5.7°C to 16.5°C, with an average range of 8.4°C.

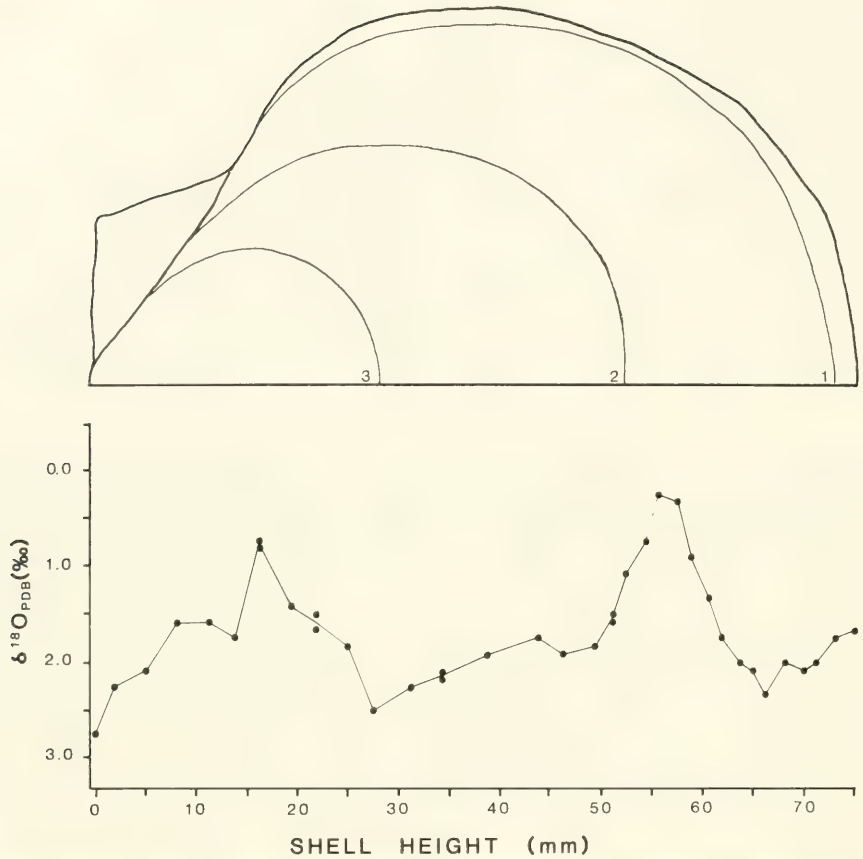

FIGURE 4. Position of external lines and shell carbonate oxygen isotopic record for the shell of specimen PM10. The external lines illustrated in the diagram are those interpreted by NMFS personnel as representing years of growth. These are numbered in reverse chronological order (line 1 is the most recently formed) to facilitate comparison with those on specimen PM26. The isotopic values of discrete sample powders are plotted with the $\delta^{18}O$ scale reversed so that lower δ values, which represent "warm" isotopic temperatures, are at the top of the vertical scale. The millimeter scale on the horizontal axis relates the position of the external lines and the position of individual carbonate powder samples to shell height.

DISCUSSION

Interpretation of observed oxygen isotope records

With the predictive model outlined previously, the oxygen isotope records obtained from the scallop shells may be interpreted as yearly cycles controlled by seasonal hydrographic conditions. The isotopic record from specimen PM10 is interpreted as showing two full years of growth with the beginning of a third year (Fig. 4). Specimen PM10 completed one year of growth at a shell height of 25 mm as determined by one full cycle in the isotopic record. Shell deposited from 0 to 10 mm gradually becomes isotopically lighter (more negative), representing late winter to late spring, and reaches an inferred late summer maximum temperature at approximately 15 mm shell height (Fig. 4). The seasonal trend continues into the late fall and early winter as represented by shell deposited from 15 to 25 mm. A second annual cycle

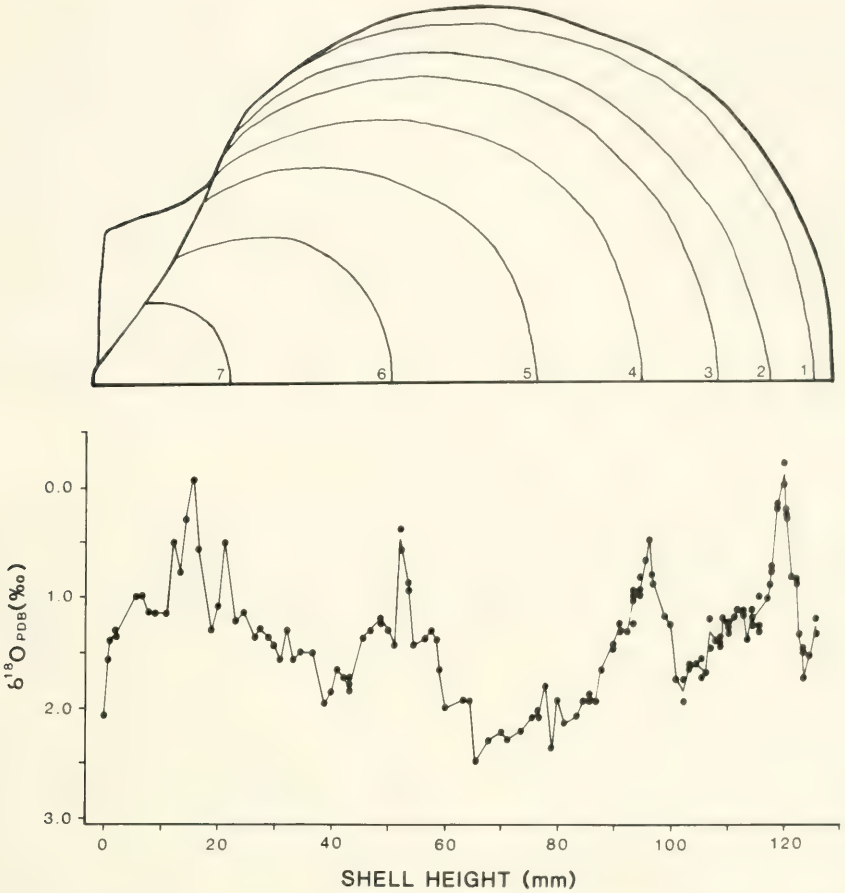

FIGURE 5. Position of external lines and shell carbonate oxygen isotopic record for specimen PM26. The external lines illustrated in the diagram are those which were interpreted by NMFS personnel as representing years of growth. As in Figure 4 for specimen PM10, the lines are numbered in reverse chronological order, and the oxygen isotopic values for shell carbonate samples are plotted with a reversed $\delta^{18}O$ scale.

begins at 30 mm, reaches a late summer temperature maximum at approximately 55 mm, and proceeds to a yearly minimum at 65 mm shell height. The beginning of a third annual cycle represented by the last 10 mm of shell and terminating at the ventral margin coincides well with the spring collection date (Fig. 4). Not only does the beginning of the isotopic cycle coincide with the seasonal hydrographic cycle, but the $\delta^{18}O$ values of samples taken from the shell margin are very close to the 1.7‰ value predicted by the model for May (Fig. 3). This agreement between isotopic "season" and collection date supports the validity of the isotopic record interpretation.

These results indicate a discrepancy between the three years of growth interpreted from the external lines and the two years of growth inferred from the $\delta^{18}O$ record. Further, the external lines, which are presumed to be deposited annually in the spring, appear to occur in various seasons according to the inferred seasonality of the isotopic profile. For example, external line number 3 was deposited in the fall of the first growth year, line 2 during summer of the second year, and line 1 the following spring (Fig. 4). In this particular specimen, there does not appear to be a consistent season

for external line formation, therefore, the assumption that these external lines represent true annual events does not appear to be valid.

As in the smaller specimen, the isotopic record from the shell of PM26 demonstrates what appear to be distinct, hydrographically controlled, annual $\delta^{18}O$ cycles (Fig. 5). Again, the four years of growth are recorded in PM26 by the cycles in the isotope curve. This age estimate is at variance with the seven years of growth interpreted from the external lines. The occurrence of external lines on the shell of PM26 appears to have a more consistent relationship to the seasonal $\delta^{18}O$ cycles than in PM10. Counting backwards from the ventral shell margin, and hence backwards chronologically, external lines 2, 4, 6, and 7 of specimen PM26 were deposited during late summer maximum temperatures (Fig. 5). A little more in line with the presumed deposition of annual lines in the spring by *Placopecten,* external lines 1, 3, and 5 appear to have been deposited in middle spring to possibly early summer. As in specimen PM10, the termination of the isotopic record at the shell margin is consistent with the spring collection date.

Comparison of the records from the two specimens

Since both scallop specimens were collected alive in 1979, calendar years may be assigned to years of growth by counting backwards from the shell margin (which represents the collection date). By assigning calendar years in this manner, the interpretation of the isotopic records and the external growth lines from the two specimens may be compared directly. In Figure 6 the shell records of specimens PM10 and PM26 are overlaid based on distance from the shell margin and on an interpretation of the isotopic record.

The cycles in the isotopic record of specimen PM26 which we interpret as representing the calendar years 1978 and 1979 do not include the isotopically heavy carbonate which the model predicts should be deposited during the winter. In comparison, the isotopic record for these same years from specimen PM10 includes carbonate isotopic values as heavy as those predicted by the model. Since other bivalves have been documented to slow or cease calcification during the winter (Taylor and Venn, 1978; Clark, 1979; Jones, 1980), it is reasonable to assume that the values "missing" from the isotopic record of specimen PM26 represent cessation of shell deposition. Similar interpretations have been proposed for the attenuation of cycles in the isotopic record for conchs (Epstein and Lowenstam, 1953; Wefer and Killingley, 1980) and surf clams (Jones *et al.,* 1983). The isotopic record of specimen PM10 is used in Figure 6 to represent the complete record for the years 1978 and 1979, while the isotopic record of specimen PM26 has been separated to illustrate periods of cessation of shell deposition. The first two years of growth in specimen PM26 appear to be relatively complete. However, the isotopic values representing the winter months of 1975–76 are slightly lighter than expected which may indicate lack of shell deposition for a short period. Alternatively, the winter of 1975–76 may have been slightly warmer than 1976–77, but this possibility can be confirmed without more complete hydrographic information.

Overall, the two major cycles in the isotopic record for specimen PM10 coincide well with the cycles from specimen PM26. The records from both shells have very similar amplitudes and absolute isotopic values. As previously discussed, the predicted $\delta^{18}O$ values demonstrate a distinct seasonality in varying from 2.5‰ in the winter to 0.5‰ in the late summer. The cycles in the isotopic records of both specimens fall almost exactly within the predicted range estimated from the hydrographic data. The isotopic temperature range of 5.7°C to 16.5°C calculated from shell calcite $\delta^{18}O$

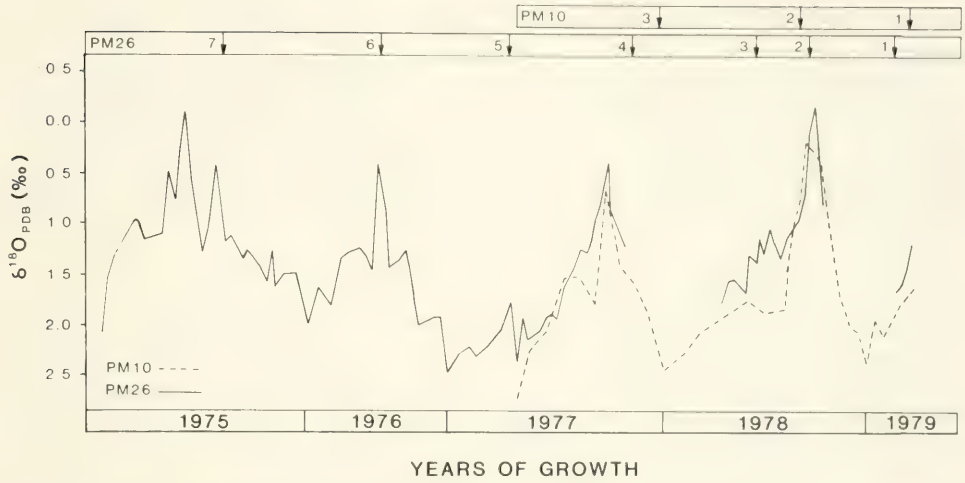

FIGURE 6. Comparison of the $\delta^{18}O$ records of specimens PM10 and PM26. The isotopic profile of specimen PM10 is represented by the dashed line, that of specimen PM26 by the solid line. Calendar years assigned to each annual cycle in the $\delta^{18}O$ records of each specimen comprise the horizontal axis. External lines interpreted as annual marks are represented for each specimen at the top of the diagram. Breaks in the record of specimen PM26 correspond to presumed curtailment of calcification by the scallop.

values corresponds well to the observed water temperatures for the area. The few excursions outside of the predicted range represent minimum and maximum temperatures which were probably dampened in the model by using hydrographic data which were averaged by month. These calculated temperatures also seem reasonable in that maximum temperatures fall below the reported lethal temperature of approximately 20°C (Dickie, 1958).

Seasons and years determined from the isotopic record allow correlation of growth lines between the two specimens and aid in evaluating possible mechanisms for line formation. External line 1 on the shell of specimen PM10 was formed in the spring before the collection date in May, 1979. Line 2 on specimen PM10 appears to correspond with line 2 on specimen PM26, both having been deposited in summer 1978 just prior to the annual temperature maximum. Similarly, growth lines 6 and 7 on specimen PM26 were deposited in middle to late summer of years 1976 and 1975 respectively.

The previously mentioned external lines appear to have been formed with little cessation of growth as evidenced by loss of "time" from the isotopic record. In contrast, external lines 1 and 4 in specimen PM26, and line 3 in specimen PM10 appear to coincide with periods of missing isotopic values. As was pointed out previously, two gaps in the isotopic record of specimen PM26 corresponding to the winters of 1978 and 1979 are interpreted as cessation of shell growth. Each of these gaps is marked by a line on the shell. On specimen PM10, line 3 coincides with a sudden shift in isotopic values, which suggests that it may be analogous to line 4 in specimen PM26 and was probably formed at approximately the same time. In each case, the specimens appear to have stopped or drastically slowed calcification in the fall and added no shell material during the winter, hence there are no isotopic values representing those periods. After this cessation of growth, specimen PM10 appears to have resumed calcification much sooner than PM26, which explains the difference in the two isotopic records for the early spring of 1978. Specimen PM10 apparently

did not curtail calcification in the winter of 1978–79, as evidenced by a fairly complete isotopic record for the period.

Sea scallops are sensitive to disturbance or abnormal changes in environmental conditions (Merrill *et al.*, 1965). External line 5 on the shell of specimen PM26 seems to coincide with anomalously light isotopic values during the early spring of 1977. The lighter than expected isotopic values were possibly the result of a low salinity event which may have eventually caused mantle retraction, shell closure, and a temporary halt to calcification.

Growth rate determinations

Since the isotopic composition of *Placopecten* appears to be controlled by seasonal hydrographic factors, the isotopic record from a shell should provide a useful time scale for determining rate of growth. The number of months from the beginning of shell deposition may be estimated for obvious seasonal peaks such as summer maxima and winter minima. This time scale may then be related directly to the positions on the shell of the respective samples. Constructed in this manner, a standard graph of shell height to years of growth (Fig. 7) illustrates that specimens PM10 and PM26 show an average rate of growth of approximately 35 mm per year (as interpreted from the isotopic record). The first two years of growth in both specimens show a very similar, approximately linear, growth rate even though they represent different calendar years. This supports the growth rate interpretation by suggesting a similar trend in ontogenetic development. For years two through four in specimen PM26, the rate of growth gradually slows, which is typical for many species of molluscs (Mason, 1957; Hallam, 1967; Jones *et al.*, 1978; Serchuk *et al.*, 1979).

The growth rates inferred from the isotope profiles of PM10 and PM26 can also be compared to the rates as determined using the external line aging technique (Fig. 8). Growth rate estimates based on the external lines would suggest that the two specimens grew approximately 20 mm per year for the first three years of growth. The larger specimen, PM26, shows a significant decrease in growth rate with age.

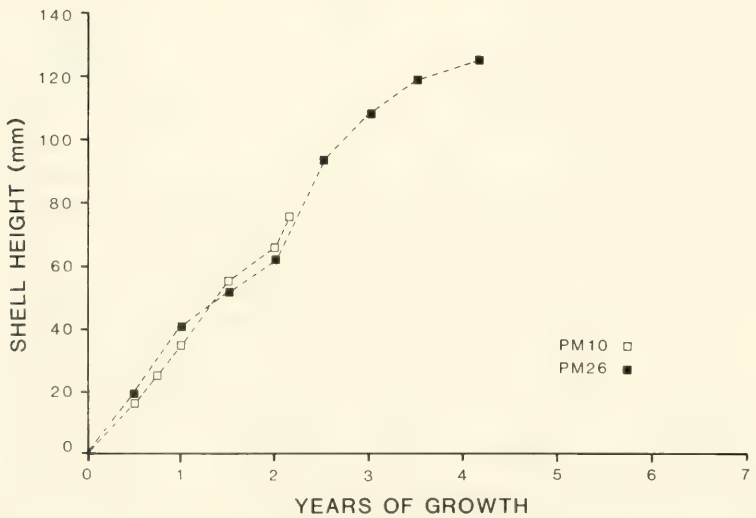

FIGURE 7. Growth rates of specimens PM10 and PM26 interpreted from the isotopic profiles. Points on each curve correspond to shell height at obvious seasonal peaks (summer maximum and winter minimum). Open squares represent shell heights for specimen PM10; closed squares represent specimen PM26.

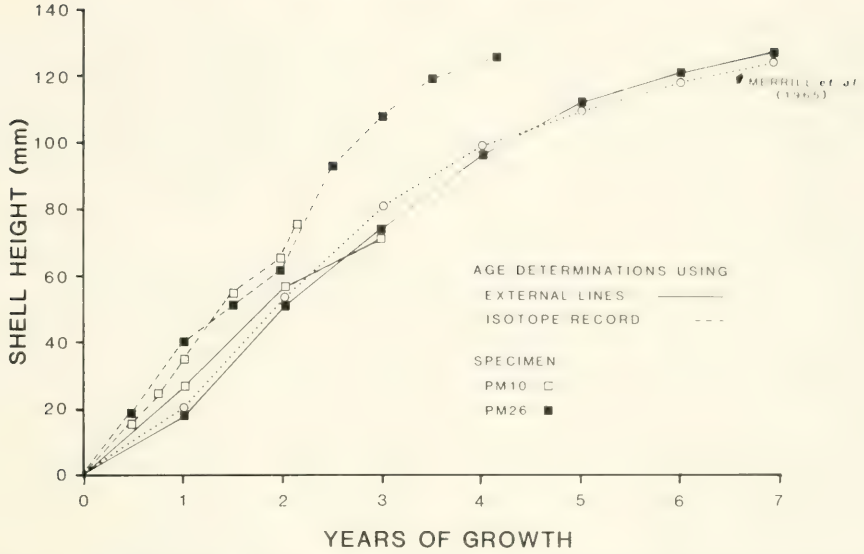

FIGURE 8. Comparison of growth rates determined from the external line technique and from the isotopic record. Growth rates for specimens PM10 and PM26 interpreted from the isotopic profiles are represented by dashed lines. Growth rates for the same two specimens determined from the external lines are represented by solid lines. Open squares indicate shell heights for specimen PM10; closed squares indicate specimen PM26. Dotted line illustrates an average growth curve for 351 *Placopecten* specimens aged using the external line method published by Merrill *et al.* (1965).

The growth rate estimates for both specimens fall very close to a curve constructed from average age/height data that Merrill *et al.* (1965) generated by estimating the age of 351 scallops using the external line technique. Although the growth rate estimates based on the external lines are internally consistent, they differ considerably from those interpreted from the isotopic record (Fig. 8). The growth rate determined from the isotopic record is roughly twice that from the external line method. This would be expected considering the discrepancy between the age (years of growth) estimates.

Migration

Results from previous tag-release studies suggest that sea scallops do not show any widespread or directed seasonal migration (Dickie, 1955; Posgay, 1963, 1981). The shell isotopic records presented here support this idea of limited movement. If the scallop specimens had migrated from either deeper or shallower water areas, the movement should have appeared as a significant change in the amplitude of the annual $\delta^{18}O$ cycles. In general on the continental shelf of the Virginia Bight, seasonal bottom water temperature fluctuations are greater in shallower water, and become progressively more dampened with increasing depth (Nickerson and Mountain, 1983). The isotopic record contained in shell carbonate, being controlled largely by ambient temperature, should also have a greater amplitude for a scallop living in shallower water. Taking into account the interruptions during calcification noted previously, specimens PM10 and PM26 have very similar isotopic amplitudes. This similarity in isotopic amplitude is seen in comparing individual cycles within the record of a single specimen, and in comparing the records of the two specimens. Therefore, it appears that the two scallop specimens, PM10 and PM26, remained in essentially the same depth habitat during their lives.

Implications for shellfisheries management

Methods presently used for the determination of age and growth rate in sea scallops rely on the interpretation of lines or rings on the shell exterior. Although some mark and recovery studies have shown that sea scallops produce an external line in the spring, lines are also produced during other times of the year, presumably in response to physical disturbance. This study demonstrates that the ratio of $^{18}O/^{16}O$ incorporated in the shell carbonate can be used to avoid the subjectivity of interpreting external lines as annual marks.

One particular advantage of using the isotopic record as an age determination tool in molluscs is the fact that the $\delta^{18}O$-derived time scale is primarily controlled by the seasonal changes in hydrographic conditions. Because of this independent control, the interpretation is less subjective than distinguishing between shock lines and true annual lines on a scallop shell. From the evaluations performed in this study, external lines do not always form concurrently or consistently for all individuals in a scallop population. On the other hand, the shell isotopic composition should record the same annual cycles for the entire population. It should, however, be noted that the stable isotope method does not lend itself to use with large numbers of specimens, primarily because of the time and expense involved in the isotopic analyses. However, the technique does allow accurate age determination for small groups of specimens used for modeling periodic external line formation, growth rates, and other processes related to biomineralization.

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LITERATURE CITED

ARTHUR, M. A., D. F. WILLIAMS, AND D. S. JONES. 1983. Seasonal temperature-salinity changes and thermocline development in the mid-Atlantic Bight as recorded by the isotopic composition of bivalves. *Geology* **11:** 655–659.

BOURNE, N. 1964. Scallops and the offshore fishery of the Maritimes. *Bull. Fish. Res. Board Can.* **145:** 1–59.

CLARK, G. R. 1979. Seasonal growth variations in the shells of recent and prehistoric specimens of *Mercenaria mercenaria* from St. Catherines Island, Georgia. *Anthropological Papers, Am. Mus. Nat. Hist.* **56:** 161–179.

COCHRAN, J. K., S. M. RYE, AND N. H. LANDMAN. 1981. Growth rate and habitat of *Nautilus pompilius* inferred from radioactive and stable isotope studies. *Paleobiology* **7:** 469–480.

DICKIE, L. M. 1955. Fluctuations in the abundance of the giant scallop *Placopecten magellanicus* (Gmelin), in the Digby Area of the Bay of Fundy. *J. Fish. Res. Board Can.* **12:** 798–856.

DICKIE, L. M. 1958. Effects of high temperatures on survival of the giant scallop. *J. Fish. Res. Board Can.* **15:** 1189–1211.

EISMA, D., W. G. MOOK, AND H. A. DAS. 1976. Shell characteristics, isotopic composition and trace-element contents of some euryhaline molluscs as indicators of salinity. *Palaeogeogr. Palaeoclimatol. Palaeoecol.* **19:** 39–62.

EPSTEIN, S., AND H. A. LOWENSTAM. 1953. Temperature-shell-growth relations of recent and interglacial Pleistocene shoal water biota from Bermuda. *J. Geol.* **61:** 424–438.

EPSTEIN, S., R. BUCHSBAUM, H. A. LOWENSTAM, AND H. C. UREY. 1951. Carbonate-water isotopic temperature scale. *Geol. Soc. Am. Bull.* **62:** 417–426.

EPSTEIN, S., R. BUCHSBAUM, H. A. LOWENSTAM, AND H. C. UREY. 1953. Revised carbonate-water isotopic temperature scale. *Geol. Soc. Am. Bull.* **64:** 1315–1326.

EPSTEIN, S., AND T. MAYEDA. 1953. Variation of ^{18}O content of waters from natural sources. *Geochim. Cosmochim. Acta* **4:** 213–224.

FAIRBANKS, R. G. 1982. The origin of continental shelf and slope water in the New York Bight: Evidence from $^{18}O/^{16}O$ ratio measurements. *J. Geophys. Res.* **87:** 5796–5808.

HALLAM, A. 1967. The interpretation of size-frequency distributions in molluscan death assemblages. *Paleontology* **10:** 25–42.

HORIBE, Y., AND T. OBA. 1972. Temperature scales of argonite-water and calcite-water systems. *Fossils* **23:** 69–79.

JONES, D. S. 1980. Annual cycle of shell growth increment formation in two continental shelf bivalves and its paleoecologic significance. *Paleobiology* **6:** 331–340.

JONES, D. S., D. F. WILLIAMS, AND M. A. ARTHUR. 1983. Growth history and ecology of the Atlantic surf clam (*Spisula solidissima*) as revealed by stable isotopes and annual shell increments. *J. Exp. Mar. Biol. Ecol.* **73:** 225–242.

JONES, D. S., I. THOMPSON, AND W. G. AMBROSE. 1978. Age and growth rate determinations for the Atlantic surf clam *Spisula solidissima*, based on internal growth lines in shell cross-sections. *Mar. Biol.* **47:** 63–70.

KEITH, M. L., G. M. ANDERSON, AND R. EICHLER. 1964. Carbon and oxygen isotopic composition of mollusk shells from marine and fresh-water environments. *Geochim. Cosmochim. Acta* **28:** 1757–1786.

KILLINGLEY, J. S., AND W. H. BERGER. 1979. Stable isotopes in a mollusk shell: detection of upwelling events. *Science* **205:** 186–188.

KRANTZ, D. E. 1983. Environmental and growth information from stable isotope records in the sea scallop, *Placopecten magellanicus*. M.S. Thesis, University of South Carolina, Columbia.

MASON, J. 1957. The age and growth of the scallop, *Pecten maximus* (L.), in Manx waters. *J. Mar. Biol. Assoc. U. K.* **36:** 473–492.

MCCREA, J. M. 1950. On the isotopic chemistry of carbonates and a paleotemperature scale. *J. Chem. Phys.* **18:** 849–857.

MERRILL, A. S. 1962. Abundance and distribution of sea scallops off the Middle Atlantic Coast. *Proc. Natl. Shellfish. Assoc.* **51:** 74–80.

MERRILL, A. S. 1971. The sea scallop. *Am. Malacol. Union Inc. Ann. Rep.* **1970:** 24–27.

MERRILL, A. S., J. A. POSGAY, AND F. E. NICHY. 1965. Annual marks on the shell and ligament of the sea scallop (*Placopecten magellanicus*). *Fish. Bull.* **65:** 299–311.

NICKERSON, S. R., AND D. G. MOUNTAIN. 1983. Surface and bottom temperature and bottom salinity distributions on the continental shelf, Cape Hatteras to Cape Sable from MARMAP cruises, 1977–1982. *MARMAP Contrib. MED/NEFC* 83-16.

PAUL, J. D. 1981. Natural settlement and early growth of spat of the queen scallop, *Chlamys opercularis* (L.), with reference to the formation of the first growth ring. *J. Molluscan Stud.* **47:** 53–58.

POSGAY, J. A. 1957. The range of the sea scallop. *Nautilus* **71:** 55–57.

POSGAY, J. A. 1963. Tagging as a technique in population studies of the sea scallop. *ICNAF Spec. Pub.* **4:** 268–271.

POSGAY, J. A. 1981. Movement of tagged sea scallops on George's Bank. *Mar. Fish. Rev.* **43:** 19–25.

SERCHUK, F. M., P. W. WOOD, J. A. POSGAY, AND B. E. BROWN. 1979. Assessment and status of sea scallop (*Placopecten magellanicus*) populations off the northeast coast of the United States. *Proc. Natl. Shellfish. Assoc.* **69:** 161–191.

STEVENSON, J. A., AND L. M. DICKIE. 1954. Annual growth rings and rate of growth of the giant scallop, *Placopecten magellanicus* (Gmelin) in the Digby Area of the Bay of Fundy. *J. Fish. Res. Board Can.* **11:** 660–671.

TAYLOR, A. C., AND T. J. VENN. 1978. Growth of the queen scallop, *Chlamys opercularis*, from the Clyde Sea area. *J. Mar. Biol. Assoc. U. K.* **58:** 687–700.

UREY, H. C. 1947. The thermodynamic properties of isotopic substances. *J. Chem. Soc.* **1947:** 562–581.

WEFER, G., AND J. S. KILLINGLEY. 1980. Growth histories of strombid snails from Bermuda recorded in their O-18 and C-13 profiles. *Mar. Biol.* **60:** 129–135.

WILLIAMS, D. F., M. A. ARTHUR, D. S. JONES, AND N. HEALY-WILLIAMS. 1982. Seasonality and mean annual sea surface temperatures from isotopic and sclerochronologic records. *Nature* **296:** 432–434.

WILLIAMS, D. F., M. A. SOMMER, II, AND M. L. BENDER. 1977. Carbon isotopic compositions of recent planktonic foraminifera of the Indian Ocean. *Earth Plan. Sci. Lett.* **36:** 391–403.

YAVNOV, S. V., AND A. V. IGNAT'EV. 1979. Shell structure and growth temperature of molluscs, family Mactridae. *Sov. J. Mar. Biol.* **5:** 409–414.

Reference: *Biol Bull.* **167**: 200–209. (August, 1984)

SELF-GENERATED *VERSUS* ENVIRONMENTALLY PRODUCED FEEDING CURRENTS: A COMPARISON FOR THE SABELLID POLYCHAETE *EUDISTYLIA VANCOUVERI*

RACHEL ANN MERZ*

Department of Anatomy, The University of Chicago, Chicago, Illinois 60637

ABSTRACT

The feeding currents produced by the branchial crown of the tube-dwelling sabellid polychaete *Eudistylia vancouveri* are compared with ambient currents experienced by the *in situ* worm. The speeds of the branchial currents range between 0.025 and 0.080 cm/s and are similar to the patterns mapped by Nicol (1930) for *Sabella pavonina.* The ambient currents in contact with the branchial crown of a worm in the field are up to three orders of magnitude higher (33.6 cm/s).

When these worms are clustered together in the field, their tubes form hemispherical mounds that affect the pattern of ambient currents. Flow over the surface of the cluster is augmented in comparison to pre-cluster velocities. Thus animals within a cluster experience higher feeding currents than do solitary worms. This increase in feeding current velocity is not without potential competition for food from clustermates. Depletion of natural particles during the passage of a single wave through a cluster ranges from 45 to 65%.

INTRODUCTION

The mechanisms by which suspension feeding animals remove particles from the surrounding fluid is a topic of current and historical interest. Fluid movement determines to a large degree the mechanical forces impinging on an organism (*e.g.,* Wainwright and Koehl, 1976; Merz, 1984), the rates of respiration and excretion (LaBarbera, 1982), and the feeding mode employed by some organisms (*e.g.,* Lewis, 1968; Warner, 1977; Tagon *et al.,* 1980; LaBarbera, 1984). Therefore, to fully and accurately understand the feeding processes and behavior of aquatic organisms, the natural flow regime of the animal in question must be taken into account (Reidl, 1971; Vogel, 1981).

Nicol (1930) describes the morphology, ciliary tracts, and feeding currents of the sabellid polychaete *Sabella pavonina.* This very detailed work is one of the most complete descriptions of the feeding mechanisms of a polychaete (Fauchald and Jumars, 1979) and has been used as a model for other studies of sabellid polychaetes (Fitzsimmons, 1965; Lewis, 1968; Bonar, 1972). It has also been incorporated into the literature as a general model for feeding in the Sabellidae (Jørgenson, 1956, 1966; Dales, 1970; Barnes, 1980). Nicol suggests that all water movement through the branchial crown of sabellids is due to ciliary activity. However, Nicol did not account for possible effects that ambient flow may have in this process. Her observations on whole worms were carried out in small closed containers of still water; finer details were ascertained by examining excised portions of branchiae.

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* Present address: Marine Science and Maritime Studies Center, Northeastern University, East Point, Nahant, Massachusetts 01908.

Dales (1957) estimated the filtration rate (volume of material strained per unit fresh worm weight) of a variety of fan worms (sabellids and serpulids) in still water by measuring changes in the optical density of graphite particles and unicellular algal suspensions. He concluded that fan worms are "clearly . . . less efficient than other suspension-feeding invertebrates, both in the volume of water they are capable of straining, and in the kind of particles which can be retained" (p. 315). However, Warner (1977) suggested that sabellids are among the suspension feeders which can use ambient water movement to augment their own self-produced currents. Because the effect of the environmental regime has never been addressed in any study of sabellids, an important aspect of the feeding mechanisms and ecology of these animals has been neglected.

Many species within Sabellidae aggregate into densely packed nonclonal mounds (Hartman, 1969). Aggregations of tube-dwellers have been shown to affect the pattern of ambient currents (Eckman, 1979, 1983; Nowell and Church, 1979). The amount of suspended material in the water may be augmented by resuspension of particles due to the presence of tubes (Eckman *et al.*, 1981; Carey, 1983) or may be depleted by the biological activity of the tube dwellers (Fager, 1964; Woodin, 1978, 1981; Levin, 1982). None of these studies has examined these phenomena for epifaunal tubes on hard substrates.

This work examines three aspects of suspension feeding in the sabellid polychaete, *Eudistylia vancouveri*. First is a comparison of the water velocities produced by the cilia of the branchial crown with the velocities of ambient currents. Second, the effect of the dense hemispherical aggregates of worms on the water flow near the feeding crown is described. Third, removal of natural particles during a single passage of water across the surface of a worm cluster in the field is quantified.

Materials and Methods

Field site and animal collection

Cattle Point, San Juan Island, Washington (48°27′N, 122°57′W) was the site for all *in situ* flow measurements, worm collection, and particle sampling. This rocky point extends into the Strait of Juan De Fuca and is one of the most exposed points in the San Juan Islands.

Specimens of *E. vancouveri* were collected by carefully peeling intact tubes away from the rock substrate during low tides. The animals were held in sea water tables with continuous circulation of fresh sea water. Only whole, undamaged worms were used for flow observation and measurement.

Flow observation and measurement

To measure and observe currents produced by cilia of the branchial crown, individual worms were supported upright in their natural tubes in a 15 × 10 × 20 cm clear plastic container and fresh sea water was circulated through the space between this inner container and an outer chamber to keep the inner box at ambient sea water temperature (10°–12°C).

Flow patterns produced by the branchial crown were visualized by releasing fluorescein sodium (uranine) dye (dissolved in sea water) at various locations around the worm. This dye was injected through PE-50 catheter tubing, the end of which had been drawn into a fine point (~300 μm diameter). The flow rate of the dye was controlled with a micrometer buret. The dye injection apparatus was mounted on a micromanipulator (for further details, see LaBarbera, 1981).

I observed the positions adopted by the worms at different ambient velocities using a recirculating flow tank with a variable speed motor (Vogel and LaBarbera, 1978). Worms in their natural tubes were arranged with the tubes extending into the center of the flow tank.

The velocity of ciliary currents in still water was measured with a thermistor flowmeter (LaBarbera and Vogel, 1976) modified to improve spatial resolution and to record very low velocities (see LaBarbera, 1981, for specific modifications and calibration procedure). Precision of velocity measurements was ±0.03 cm/s; accuracy was approximately the same. The probe was 500 μm in diameter and its spatial resolution was 0.5 mm.

Current velocities within and around *in situ* clusters were measured with a portable electromagnetic water current meter (Model 511, Marsh-McBirney, Inc.). Precision of this instrument was ±2% of reading. The probe was 2.5 cm in diameter. An adjustable aluminum scaffolding was used to hold the flow probe securely in the desired location while readings were taken. All *in situ* velocities were recorded on a Linear Model 142 portable chart recorder. All velocities reported here are the mean (±S.D.) of the peak velocities of a series of waves moving through a surge channel at a particular location.

Measurement of particle depletion

To measure removal of particles from the water by a cluster of *E. vancouveri,* water samples were taken from a single wave before and after it washed through a worm cluster. To insure that the same water mass was sampled on both sides of a cluster, fluorescein dye was released upstream from the worm. A water sample was taken immediately before this marked water moved through the worm cluster, and again as it emerged from the downstream side. During sampling the water surface was never more than 3 cm above the worm tubes. Thus, these samples represent water moving through the worm cluster at crown height.

The water samples (30–80 ml) were taken with a large bore (5 mm) suction device. Each water sample was transferred to a sterile glass bottle, sealed, and stored on ice in the dark for transport to the laboratory. Elapsed time from sample collection to particle counting was less than four hours.

Three different worm clusters were sampled in this manner. To estimate the repeatability of sampling, one cluster was sampled twice (two separate waves, 20 minutes apart). At another cluster, two downstream samples were taken from a single wave.

In the laboratory, each water sample was gravity filtered through a 102 μm Nitex filter. This filtered sample was then gently inverted several times and a 2 ml subsample was removed for measurement. The precision of counts from multiple subsamples is better than 5% (B. Best, pers. comm.). The frequency distribution for 128 particle size classes was tallied and recorded by an Elzone 80XY particle counter (Particle Data, Inc.). Particle size was measured as displacement volume and is reported as the diameter of a sphere of equivalent volume. For a description of this technique, see Haven and Morales-Alamo (1970).

RESULTS

Pattern and velocities of worm-generated currents

The pattern of water movement around a fully expanded branchial crown of *Eudistylia vancouveri* in still water agrees with Nicol's (1930) description (Fig. 1B).

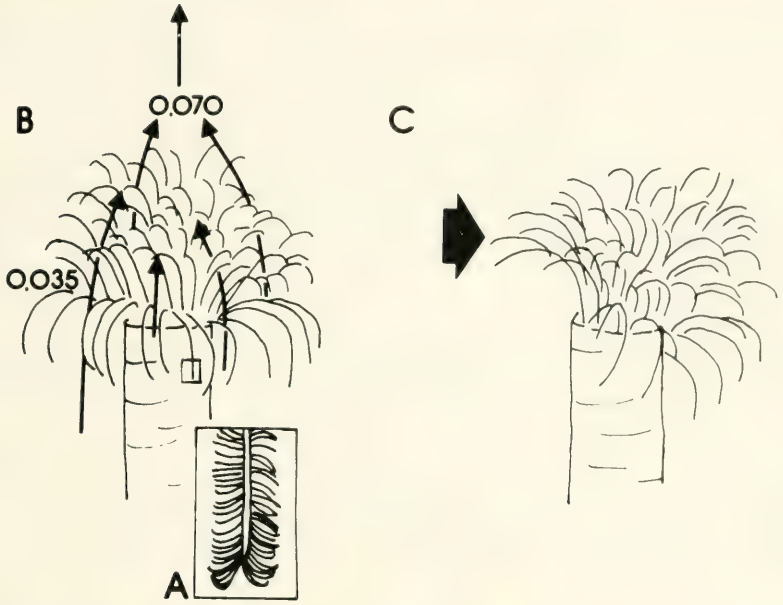

FIGURE 1. Posture of the branchial crown of *Eudistylia vancouveri* in still and moving water. (A) Enlargement of a filament and its associated pinnules. (B) Position of the filaments in still water, arrows indicate the dye streams produced by ciliary currents, the speeds of which are reported as cm/s. (C) Position of the filaments in flowing water (20 cm/s), heavy arrow shows the direction of water movement, the position of filaments and pinnules were traced from photographs.

The cilia-driven currents flow from under the branchial crown through the network of filaments. This flow is completely laminar. There is no evidence of the pulsatile flow that would result if this current were produced by the peristaltic pumping of the body within the tube. The streamlines do not mix within the branchial crown, but converge towards the midpoint above it.

Current speed at the periphery of the crown (0.5 cm above the distal tips of the filaments) ranged between 0.025 and 0.045 cm/s ($X = 0.035 \pm 0.004$ S.E.). The current speed above the center of the crown (no more than 0.5 cm from the midpoint) ranged between 0.056 and 0.080 cm/s ($X = 0.070 \pm 0.006$ S.E.). Thus the speed at the center of the crown is about twice that at the periphery. This higher speed corresponds to the larger number of cilia-covered filaments in this center region. Additionally, this central stream of higher speed may act to entrain the peripheral streamlines and result in their convergence above the branchial crown.

Crown posture in ambient currents

The filaments of the branchial crown of *E. vancouveri* are arranged in two lateral spirals of equal size (Banse, 1979). When the worm emerges from its tube these paired whorls of filaments unfurl and fill a volume above the tube that is like a rounded cone or pointed hemisphere in shape. Each filament describes an arc, with the pinnules on the upper or oral surface (Fig. 1A). In still water, the branchial crown is positioned symmetrically over the tube, with the plane of the base of the crown perpendicular to the long axis of the tube (Fig. 1B).

In flowing water, 10 to 40 cm/s, the apex of the crown is angled downstream. In this position the leading edge of the crown is raised and the plane of the base of the crown is no longer perpendicular to the long axis of the tube (Fig. 1C). The spiral tiers of the crown intersect the ambient flow at approximately 30° to 40°. This orientation of suspension feeding structures is known for a wide variety of animals (Warner, 1977). The paths of natural particles and fluorescein dye streams around the branchial crown in this orientation indicate that there is a downstream eddy into which the pinnules of the filaments project. At higher speeds (above 40 cm/s), the worms partially withdraw into their tubes, drawing the filaments of the crown together.

Flow patterns in the field

The distribution of flow velocities around a cluster indicates that the cluster acts as a semi-porous barrier or breakwater, causing the bulk of water in a passing wave to flow over the surfaces of the cluster rather than through the mass of tubes (Table I, Fig. 2). The flow speed is highest at the surface of the cluster where the branchial crowns are positioned. Lower values occur within the cluster, below the surface of the branchial crowns. There is no appreciable change in flow speeds around solitary animals in the same habitat. Thus, animals in clusters experience higher flow at crown level than do solitary animals when both are located in the same habitat.

Particle removal

The total particle depletion during a single wave passage through a cluster ranges from 45 to 65%. Figure 3 gives the size-frequency distribution of particles in a wave before and after passing a worm cluster; Figure 2 illustrates the water sampling locations. There is less than a 9% difference in this value for two waves passing over the same cluster; and less than a 7% difference for duplicate samples taken from the same wave downstream from a cluster. The fraction of particles removed was not constant over all sizes ($\alpha = 0.05$, Kolmogorov-Smirnov; Siegel, 1956). In all cases the lowest percent particle removal was at the large end of the size distribution, above 7 μm. The highest percent removal was between 3 and 6 μm.

Discussion

Two factors have led to potentially erroneous views about the method and degree of success of suspension feeding in the sabellid polychaetes. In the first case, previous

Table I

Current speeds around clustered and solitary worms[1]

	Mean maximum speed ±S.D. (N) (cm s^{-1}) pre-cluster	Mean maximum speed ±S.D. (N) (cm s^{-1}) crown level	Mean maximum speed ±S.D. (N) (cm s^{-1}) below crowns	Worm density (tubes m^{-2})	Cluster length parallel to flow (cm)
Cluster 1	11.2 ± 2.1 (13)	12.7 ± 1.0 (12)	8.0 ± 2.2 (13)	1243	50
Cluster 2	23.6 ± 2.1 (11)	31.8 ± 1.8 (11)	3.7 ± 0.8 (13)	2576	150
Cluster 3	27.7 ± 2.9 (16)	33.6 ± 2.3 (23)	16.6 ± 2.7 (17)	2448	50
Solitary worm	8.5 ± 0.7 (15)	8.5 ± 1.3 (15)	—	1	—

[1] The placement of probes around clusters is diagrammed in Figure 2.

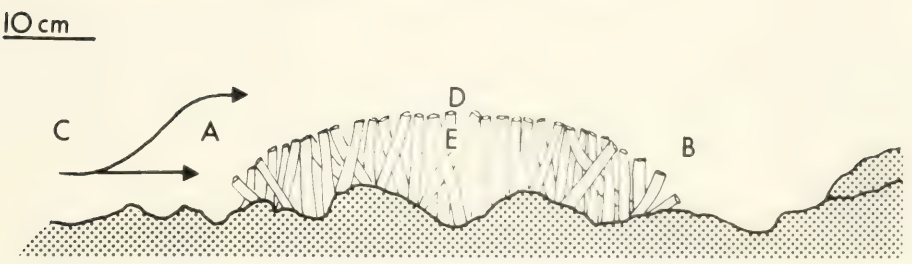

FIGURE 2. Flow and water sampling sites through a section of a cluster of *Eudistylia vancouveri*. (A) Site of pre-cluster water sample removed. (B) Site of post-cluster water sample removed. (C) Site of upstream dye release and velocity measurements. (D) Site of velocity measurements at crown height. (E) Site of below crown velocity measurements. Arrow indicates the direction of flow during water sampling and traces the path of the dye marker during one wave passing through the cluster. Note that the sampling site is in a surge channel and flow is bidirectional. Cluster drawn to scale.

workers (Nicol, 1930; Dales, 1957; Fitzsimmons, 1965; Lewis, 1968; Bonar, 1972; Sorokin, 1973) have not taken into account the importance of ambient water movement in suspension feeding. They have all studied the process in still water, a fluid regime the worms rarely, if ever, experience. However, if the natural flow conditions are considered, it is clear that the velocity of water in contact with and surrounding the branchial crown is two to three orders of magnitude greater than that produced by the cilia alone. Thus, any estimate of feeding that is based on only ciliary currents grossly underestimates the rate at which water and food are processed by the worm. Second, it is important to realize that flow rates *per se* are not the sole criterion by which filtration efficiency should be evaluated. If a suspension feeder can acquire all the food it needs by generating low filtration velocities, it may well be more efficient than suspension feeders that generate high flow rates at greater energetic cost (this latter point is addressed in LaBarbera, 1984).

In ambient flow, the majority of the feeding surface of the crown (the pinnules) is positioned on the downstream side of the worm. The wake of tubes is characterized as an area of relatively slow moving fluid, forming erratic spirals or eddies (Carey, 1983). Warner (1977) suggests that the advantage of positioning particle capture surfaces in this area is that reduced flow speed and chaotic or recirculating particle movement may enhance capture of the particles (also see Meyer, 1973; Rubenstein and Koehl, 1976).

Clusters of *E. vancouveri* remove up to 65% of all particles in a wave washing over them (more than 70% of particles 3 to 6 μm in diameter, Fig. 3). Sabellids are known to filter bacterial cells 0.5 μm in diameter from a suspension of single cells. Only sponges were shown to be as successful at this small size range (Sorokin, 1973). Other suspension feeders (ascidians, bivalves, and calanoid copepods) take larger particles (above 3 to 7 μm in diameter) (Haven and Morales-Alamo, 1970; Sorokin, 1973; Vahl, 1973; Wright *et al.*, 1982). Thus it may be that sabellids are concentrating on the smaller range of plankton composed of bacterio-, myco-, and small phytoplankton, which are readily assimilated (Sorokin, 1973) and constitute the greatest proportion of the planktonic biomass (Sieburth *et al.*, 1978).

The densely packed mounds of tubes of *E. vancouveri* alter the velocity profile of an incoming wave. The thicket of tubes restricts water flow and forces the bulk of the water to travel over the surface of the mound. This flow pattern has two advantages for the worms. The tubes are subjected to lower drag forces (Merz, 1984)

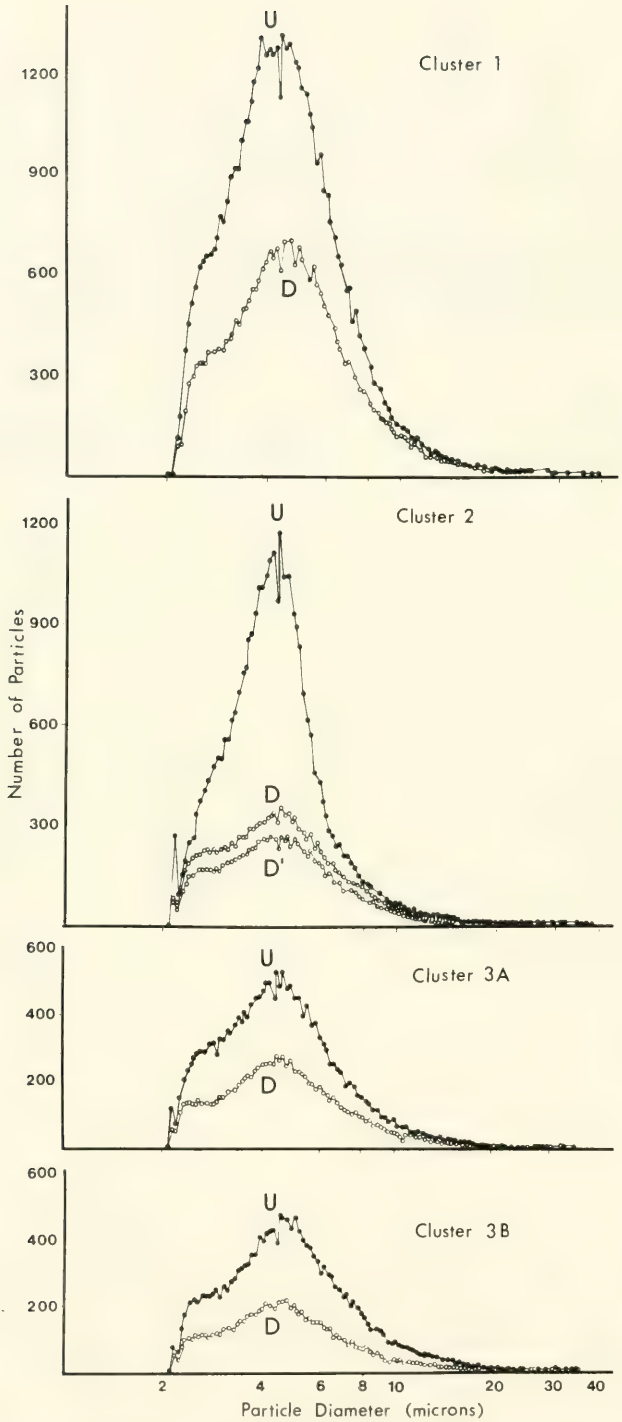

FIGURE 3. Size-frequency distributions for natural suspended particles before and after passing through a worm cluster. In each case line U represents the upstream pre-cluster sample, line D or D′ represent the post-cluster sample (see Fig. 2). The results from three clusters are shown. In cluster 2, lines D and D′ represent two post-cluster samples taken from the same wave. Cluster 3A, B are measurements of the same cluster for two different waves, 20 minutes apart. (These clusters are not those shown in Table I).

and the feeding crowns are positioned in the fastest flowing water at the surface of the cluster (Fig. 2, Table I) where they are exposed to more water and food per unit time than is a solitary animal (Table I). Koehl (1977) suggests that sessile suspension feeders should minimize drag on their support structure while maximizing flow through their feeding structure. The hemispherical aggregates of *E. vancouveri* accomplish both of these objectives.

Many suspension feeders live with non-clonal conspecifics in hemispherical mounds. For example, sabellid genera contain species that aggregate in this way (O'Donoghue, 1924; Chapman, 1955; Hartman, 1969; Koechlin, 1977), as do some chaetopterid polychaetes (Bailey-Brock, 1979), phoronids (Johnson, 1959; Ronan, 1975), and goose barnacles (Kozloff, 1973). All these animals are either passive suspension feeders or are facultatively active suspension feeders (*sensu* LaBarbera, 1977), and as such depend to some extent on ambient flow for feeding. These animals cluster together into potentially competitive aggregates for several reasons. One may be that domes increase flow through the feeding structures and simultaneously reduce drag on supportive structures. The mound itself forces the bulk of water to flow over the surface. Thus each animal is acting in its own interests, but in so doing contributes to the formation that benefits others in the mound.

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LITERATURE CITED

BAILEY-BROCK, J. H. 1979. Sediment trapping by chaetopterid polychaetes on a Hawaiian fringing reef. *J. Mar. Res.* **37:** 643–656.

BANSE, K. 1979. Sabellidae (Polychaeta) principally from the Northeast Pacific Ocean. *J. Fish. Res. Board Can.* **36:** 869–882.

BARNES, R. D. 1980. *Invertebrate Zoology.* W. B. Saunders Co., Philadelphia. 1089 pp.

BONAR, D. B. 1972. Feeding and tube construction in *Chone mollis* Bush (Polychaeta, Sabellidae). *J. Exp. Mar. Biol. Ecol.* **9:** 1–18.

CAREY, D. A. 1983. Particle resuspension in the benthic boundary layer induced by flow around polychaete tubes. *Can. J. Fish. Aquat. Sci.* **40**(Suppl.): 301–308.

CHAPMAN, G. 1955. Aspects of the fauna and flora of the Azores. VI. The density of animal life in the coralline alga zone. *Ann. Nat. Hist.* **51:** 801–805.

DALES, R. P. 1957. Some quantitative aspects of feeding in sabellid and serpulid fan worms. *J. Mar. Biol. Assoc. U.K.* **36:** 309–316.

DALES, R. P. 1970. *Annelids.* Hutchinson University Library, London. 200 pp.

ECKMAN, J. E. 1979. Small-scale patterns and processes in a soft-substratum, intertidal community. *J. Mar. Res.* **37:** 437–457.

ECKMAN, J. E. 1983. Hydrodynamic processes affecting benthic recruitment. *Limnol. Oceanogr.* **28:** 241–257.

ECKMAN, J. E., A. R. M. NOWELL, AND P. A. JUMARS. 1981. Sediment destabilization by animal tubes. *J. Mar. Res.* **39:** 361–374.

FAGER, E. W. 1964. Marine sediments: Effects of a tube-building polychaete. *Science* **143:** 356–359.
FAUCHALD, K., AND P. A. JUMARS. 1979. The diet of worms: a study of polychaete feeding guilds. *Oceanogr. Mar. Biol. Ann. Rev.* **17:** 193–284.
FITZSIMMONS, G. 1965. Feeding and tube-building in *Sabellaestarte magnifica* (Shaw) (Sabellidae; Polychaeta). *Bull. Mar. Sci.* **15:** 642–671.
HARTMAN, O. 1969. *Atlas of the Sedentariate Polychaetous Annelids from California.* Allan Hancock Foundation, Los Angeles, California. 812 pp.
HAVEN, D. S., AND R. MORALES-ALAMO. 1970. Filtration of particles from suspension by the American oyster *Crassostrea virginica. Biol. Bull.* **139:** 248–264.
JOHNSON, R. G. 1959. Spatial distribution of *Phoronopsis viridis* Hilton. *Science* **129:** 1221.
JØRGENSON, C. B. 1955. Quantitative aspects of filter feeding in invertebrates. *Biol. Rev.* **30:** 391–454.
JØRGENSON, C. B. 1966. *Biology of Suspension Feeding.* Pergamon Press, Oxford. 357 pp.
KOECHLIN, N. 1977. Installation d'une épifauna á *Spirographis spallanzani* Viviani, *Sycon ciliatum* Favricius et *Ciona intestinalis* (L.) dans le port de plaisance de Lézardrieux (Côtes-du-Nord). *Cahiers de Biol. Mar.* **18:** 325–337.
KOEHL, M. A. R. 1977. Effects of sea anemones on the flow forces they encounter. *J. Exp. Biol.* **69:** 87–105.
KOZLOFF, E. N. 1973. *Seashore Life of Puget Sound, the Strait of Georgia, and the San Juan Archipelago.* University of Washington Press, Seattle. 282 pp.
LABARBERA, M. 1977. Brachiopod orientation to water movement. 1 Theory, laboratory behavior, and field orientations. *Paleobiology* **3:** 270–287.
LABARBERA, M. 1981. Water flow patterns in and around three species of articulate brachiopods. *J. Exp. Mar. Biol. Ecol.* **55:** 185–206.
LABARBERA, M. 1982. Metabolic rates of suspension feeding crinoids and ophiuroids (Echinodermata) in a unidirectional laminar flow. *Comp. Biochem. Physiol.* **71A:** 303–307.
LABARBERA, M. 1984. Feeding currents and capture mechanisms in suspension feeding animals. *Am. Zool.* **24:**(1): 71–84.
LABARBERA, M., AND S. VOGEL. 1976. An inexpensive thermistor flowmeter for aquatic biology. *Limnol. Oceanogr.* **21:** 750–756.
LEVIN, L. A. 1982. Interference interactions among tube-dwelling polychaetes in a dense infaunal assemblage. *J. Exp. Mar. Biol. Ecol.* **65:** 107–119.
LEWIS, D. B. 1968. Feeding and tube-building in the Fabriciinae (Annelida, Polychaeta). *Proc. Linn. Soc. Lond.* **179:** 37–49.
MERZ, R. A. 1984. An experimental field study of the role of flow on the feeding behavior, tube structure, and cluster morphology of the sabellid polychaete *Eudistylia vancouveri.* Ph.D. Dissertation, Univ. Chicago.
MEYER, D. L. 1973. Feeding behavior and ecology of shallow-water unstalked crinoids (Echinodermata) in the Caribbean Sea. *Mar. Biol.* **22:** 105–129.
NICOL, E. A. T. 1930. The feeding mechanism, formation of the tube, and physiology of digestion in *Sabella pavonina. Trans. R. Soc. Edin.* **56:** 532–598.
NOWELL, A. R. M., AND M. CHURCH. 1979. Turbulent flow in a depth-limited boundary layer. *J. Geophys. Res.* **84:** 4816–4824.
O'DONOGHUE, C. H. 1924. A note on the polychaetous annelid *Eudistylia gigantea,* Bush. *Contrib. Can. Biol. Fish.* **1**(N.S.): 443–453.
REIDL, R. 1971. Water movement, animals. Pp. 1123–1156 in *Marine Ecology,* Vol. I., Part 2, O. Kinne, ed. Wiley Interscience, New York.
RONAN, T. E., JR. 1975. Structural and paleoecological aspects of a modern marine sediment community: An experimental field study. Ph.D. Dissertation, Univ. California, Davis.
RUBENSTEIN, D. I., AND M. A. R. KOEHL. 1976. The mechanisms of filter feeding: Some theoretical considerations. *Am. Nat.* **111:** 981–999.
SIEBURTH, J. MCN., V. SMETACEK, AND J. LENZ. 1978. Pelagic ecosystem structure: Heterotrophic compartments of the plankton and their relationship to planktonic size fraction. *Limnol. Oceanogr.* **23:** 1256–1263.
SIEGEL, S. 1956. *Nonparametric Statistics for the Behavioral Sciences.* McGraw-Hill, New York. 312 pp.
SOROKIN, Y. I. 1973. Microbiological aspects of the productivity of coral reefs. Pp. 17–45 in *Biology and Geology of Coral Reefs,* Vol. II., Biology 1, O. A. Jones, and R. Endeon, eds. Academic Press, New York.
TAGON, G. L., A. R. M. NOWELL, AND P. A. JUMARS. 1980. Induction of suspension feeding in spionid polychaetes by high particulate fluxes. *Science* **210:** 562–564.
VAHL, O. 1973. Efficiency of particle retention in *Chlamys islandica* (O. F. Muller). *Astarte* **6:** 21–25.
VOGEL, S. 1981. *Life in Moving Fluids.* Willard Grant Press, Boston, Massachusetts. 352 pp.

VOGEL, S., AND M. LABARBERA. 1978. Simple flow tanks for research and teaching. *Bioscience* **28:** 638–643.

WAINWRIGHT, S. A., AND M. A. R. KOEHL. 1976. The nature of flow and the reaction of benthic cnidaria to it. Pp. 5–21 in *Coelenterate Ecology and Behavior*, G. O. Mackie, ed. Plenum Press, New York.

WARNER, G. 1977. On the shapes of passive suspension feeders. Pp. 567–576 in *Biology of Benthic Organisms*, Eleventh European Symposium on Marine Biology, B. F. Keegan, P. O. Ceidigh, and P. J. S. Boaden, eds. Pergamon Press, Oxford.

WOODIN, S. A. 1978. Refuges, disturbance, and community structure: A marine soft-bottom example. *Ecology* **59:** 274–284.

WOODIN, S. A. 1981. Disturbance and community structure in a shallow water sand flat. *Ecology* **62:** 1052–1066.

WRIGHT, R. T., R. B. COFFIN, C. P. ERSING, AND D. PEARSON. 1982. Field and laboratory measurements of bivalve filtration of natural marine bacterio-plankton. *Limnol. Oceanogr.* **27:** 91–98.

Reference: *Biol. Bull.* **167**: 210–228. (August, 1984)

GIANT SMOOTH MUSCLE FIBERS OF THE CTENOPHORE *MNEMIOPSIS LEYDII:* ULTRASTRUCTURAL STUDY OF *IN SITU* AND ISOLATED CELLS

MARI-LUZ HERNANDEZ-NICAISE, GHISLAIN NICAISE, AND LUC MALAVAL

Histologie et Biologie Tissulaire, Université Claude Bernard, 43 Boulevard du 11 Novembre, 69622—Villeurbanne, France[1]

ABSTRACT

The lobate ctenophore *Mnemiopsis leydii* possesses giant smooth muscle fibers grouped in two sagittal bundles. Functional isolated cells were obtained by an enzymatic digestion of mesoglea and epithelia.

Each bundle is made of 30 to 50 multinucleated cylindrical cells which may reach 35 μm in diameter and 4 cm in length. The nuclei and non-contractile organelles (mitochondria, golgi, rough endoplasmic reticulum) are contained in a discontinuous axial core, surrounded by a thick sheath of myofilaments. Thin (actin) filaments, 5.9 nm in diameter, form irregular rosettes around the thick (myosin) filaments, 16.1 nm in diameter. An actin:myosin filament ratio of 7:2 and a myosin density of 249 filaments per μm^2 were found in cross-sections of relaxed *in situ* cells. No dense bodies nor attachment plates were observed. From the coiled shape of contracted single cells and from the rearrangement of organelles in such coiled cells, we propose that myofilaments are organized in thin long myofibrils attached to the cell membrane at both ends, and that the attachment sites follow two (sets of) enantiomorphic helices. The sarcoplasmic reticulum is a longitudinally oriented 3-dimensional network of narrow tubules among the myofilaments. Its relative volume, estimated from cross sections, amounts to 0.9% of the contractile cytoplasm. No peripheral couplings have been observed, nor any tubular or vesicular invagination of the sarcolemma.

INTRODUCTION

The first giant smooth muscle fiber so far known has been described in a planktonic invertebrate, the ctenophore *Beroe* (Hernandez-Nicaise and Amsellem, 1980; Hernandez-Nicaise *et al.*, 1980). These multinucleated cells can reach several centimeters in length and 50 μm in diameter; they have no striation, dense bodies or attachment plates, at least when observed after conventional processing for electron microscopy.

A simple technique using hyaluronidase and trypsin has been performed to isolate functional muscle cells of *Beroe.* Their ultrastructure and the electrical properties of their membrane are similar to those of *in situ* fibers (Hernandez-Nicaise *et al.*, 1982). Such a single giant smooth muscle fiber provides a multi-purpose model for cell biological studies of smooth muscle. Recent studies using a single cell preparation from a lower vertebrate (Bagby *et al.*, 1971) have already lead to a better understanding of smooth muscle cell organization and function (Fay, 1976; Walsh and Singer, 1981; Fay *et al.*, 1983).

These possibilities are hindered by the limited availability of *Beroe.* To our knowledge, this species is available along the French Mediterranean coast (Station Zoologique at Villefranche-sur-Mer) only from March to May, and as the animals feed upon

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[1] Present address: Cytologie expérimentale, Université de Nice, Parc Valrose, 06034 Nice, France.

other fragile planktonic species they cannot be kept in good condition for more than one or two weeks in the laboratory. We thus tried to identify another species which could (1) yield giant smooth muscle fibers, (2) be available over long periods at low cost, and (3) be kept in a marine laboratory without excessive care.

We found that the lobate species *Mnemiopsis leydii* (Fig. 1) meets all these requirements: it is possible to isolate functional giant smooth muscle fibers from the two sagittal muscle bundles; it is an ubiquitous neritic species of the southern shores of North America and is, for example, common throughout the summer in Woods Hole, Massachusetts; and the animals can be kept several days in still sea water, renewed every day. The possibility of rearing closely related species through long periods has been demonstrated (Baker and Reeve, 1974; Ward, 1974).

The present report describes the method used to obtain functional single muscle cells and gives a detailed electron microscopic description of the *in situ* and isolated cell together with a stereological analysis of the sarcoplasmic reticulum (SR). The ultrastructural features of *Mnemiopsis* giant smooth muscle cells are compared with those of *Beroe*.

A preliminary electro-physiological investigation has been conducted prior to this morphological study to ascertain the physiological integrity of freshly isolated cells

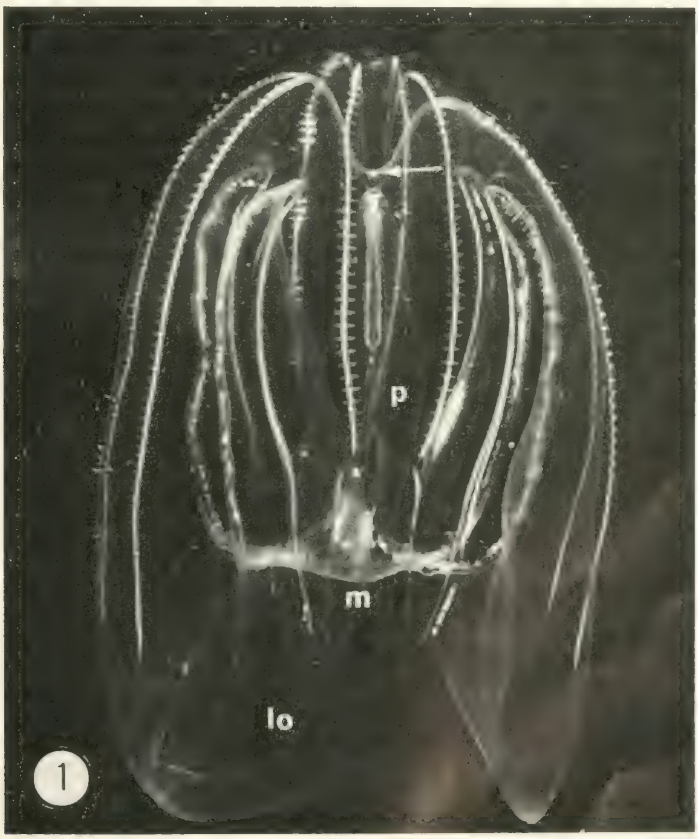


FIGURE 1. Photograph of a living *Mnemiopsis leydii*, viewed from the tentacular side. The animal is swimming downward with mouth (m) and oral lobes (lo) below and aboral organ (arrow) uppermost. The pharynx (p) is visible through the transparent mesoglea. 1.5×.

(Hernandez-Nicaise *et al.*, 1981) and has shown that the resting potentials (average: −56 mV), membrane impedances, and action potentials of isolated and *in situ* fibers do not differ significantly. Further electrophysiological studies are in progress (Anderson, in press).

Materials and Methods

Specimens of *Mnemiopsis leydii* were collected with plastic beakers along the jetty of the N. M. Fisheries Service, Woods Hole, Massachusetts. The two sagittal muscle bundles (Fig. 2) were mechanically removed from the animals. Care was taken to eliminate as much mesoglea as possible, and to preserve the attachment of the muscle cells at both ends (endodermic lining of aboral canal and epidermis of the lips). The dissected bundles were kept in cooled artificial sea water (ASW) prepared according to Cavanaugh (1956) (NaCl, 423 m*M;* KCl, 9 m*M;* $CaCl_2$, 9 m*M;* $MgCl_2$, 23 m*M;* $MgSO_4$, 26 m*M;* $NaHCO_3$, 2 m*M*).

Cell preparation

The procedure devised for *Beroe* muscles (Hernandez-Nicaise *et al.*, 1982) appeared poorly adapted for *Mnemiopsis* muscular bundles, and was modified as follows: after a brief rinse in Ca-free ASW, the dissected bundles were pre-incubated at 30°C for 75–90 min in 0.3% hyaluronidase (type III, Sigma) in nominally Ca-free ASW. After this incubation the mesoglea surrounding the muscle cells was considerably softened and could be removed by dissection.

The tissues were then incubated in 0.05% trypsin (type III, Sigma) and 0.3% hyaluronidase at 30°C for 20–30 min in Ca-free ASW. The bundles were then transferred to Ca-free ASW. They were gently agitated by blowing them in and out of a large bore siliconized Pasteur pipette, until the individual cells were freed from the bundle. The released muscle cells were transferred to cold Ca-containing ASW. From this stage on, siliconized glassware was used.

Electron microscopy

Freshly dissected bundles could not be fixed by immersion into a fixative. Observation of the bundles under the microscope during the onset of such fixation showed that the contact of the extremities of muscle fibers with the fixative triggers such a violent contraction that the muscles break into small fragments and disintegrate.

To prevent this contraction two alternative protocols were devised: (1) the dissected bundles were incubated for 30 min in 0.3% hyaluronidase in Ca-free ASW prior to fixation; the cells remained relaxed when immersed in fixative, or (2) the bundles were incubated for one hour in Ca-free ASW prior to fixation; in this case the cells had an undulated appearance probably due to an interaction between the remaining intact mesoglea and the fixative. All the figures of *in situ* fibers presented in this study were obtained from bundles pretreated with hyaluronidase.

Single cells were transferred by pipette into fixative without any damage.

Bundles and single cells were fixed with 5% glutaraldehyde in cacodylate-buffered ASW (pH 7.7) at room temperature. Following a brief rinse in this buffered isotonic saline, the cells or the whole bundles were post-fixed in 2% osmium tetroxide in the same saline. Some specimens were block-stained with 1% tannic acid (Mallinckrodt) in 0.1 *M* sodium cacodylate (pH 6.5). The specimens were subsequently dehydrated in a graded series of ethyl alcohol followed by three changes in propylene oxide and embedded in durcupan. Silver grey sections were stained with uranyl acetate followed by lead citrate.

Morphometry

Six bundles from different individuals were cut transversally. An electron micrograph at an initial magnification of 10,000× was taken from sections of seven fibers randomly chosen in each bundle. The magnification of the electron microscope was repeatedly calibrated against an optical grating replica (1260 lines/mm). Measurements were performed on prints at an exact magnification of 30,000×, with an image analyzer Videoplan (Kontron, Germany). The total surface of each muscle fiber section and the surface occupied by the axial column and the mitochondria were measured. The difference between these two areas was defined as the contractile cytoplasm area ("Acc").

The area, "Asr," and membrane length, "Bsr," of sarcoplasmic reticulum (SR) were also measured and the number of SR profiles, "Nsr," was counted.

Our hypothesis was that the morphology of the SR (see Results) is consistent with a stereological model of continuous tubules parallel to the main axis of the fiber, as defined by Weibel (1972) for the SR of striated muscle fibers. Under these conditions, the relative area, Asr/Acc, and the relative membrane length, Bsr/Acc, of the SR on a given section are representative of the relative volume, Vv; and if the section is transverse, they are representative of the relative surface, Sv, of the SR in the contractile cytoplasm (Weibel, 1972). These stereological parameters were estimated, after a preliminary statistical study, by averaging the calculated ratios of each section (estimator 1 of case II in Cruz-Orive, 1980), thus: $Vv = \frac{1}{n} \Sigma \frac{Asr}{Acc}$, $Sv = \frac{1}{n} \Sigma \frac{Bsr}{Acc}$, n being the number of sections of measured fibers. In the case of Sv, the value obtained by assuming an isotropic organization $Sv = \frac{4}{\pi n} \Sigma \frac{Bsr}{Acc}$, has also been calculated. The numerical density of the SR tubules in sections was estimated in the same way by $NA = \frac{1}{n} \Sigma \frac{Nsr}{Acc}$.

Quantitative study of myofilaments

The densities and diameters of myofilaments were estimated from micrographs taken from the same sample as above. Measurements were performed on prints at a final magnification of 100,000×.

The number of myosin filaments per μm^2 was counted on cross sections of 14 cells from 4 bundles. A one way variance analysis showed that there was no significant differences between two fields within a same section, between two cells of the same bundle, nor between two cells of different animals.

Therefore a smaller sample was used to determine actin density (4 cells) and filaments diameters (4 cells, 50 filaments of each category per cell).

Freeze-fracturing

For freeze-fracturing studies whole bundles were fixed at Woods Hole in 5% glutaraldehyde in cacodylate buffered, Ca-free ASW. They were kept in ice-cold fixative for several days (3 to 7) until they were processed in our laboratory in France. The tissue samples were thoroughly rinsed in several baths of buffered saline and dissected into small pieces. The specimens were infiltrated with 30% glycerol in the same buffer for 3 hours. Tissue blocks were then mounted between two copper discs, rapidly frozen at −210°C in nitrogen slush, and placed into the double-fracture device of a "Reichert Jung CF 250" unit. They were then fractured at a stage temperature of

−150°C, under a vacuum of 1.10^{-8} Torr without etching. The exposed surfaces were shadowed with carbon/platinum at an angle of 45°. Replicas were cleaned with sodium hypochlorite, repeatedly rinsed in distilled water, and mounted on copper grids.

RESULTS

Light microscope observations on living bundles

The mesoglea of *Mnemiopsis leydii* is crossed by numerous muscle fibers. Most of them are too widely separated from each other and too small in diameter to be interesting either for *in situ* studies or as single cell preparations as is the case for *Beroe* muscle cells. Two sagittal bundles of thick long muscle fibers are known to occur in *Mnemiopsis* (Fig. 2) as well as in other lobates (Chun, 1880), but have been paid little attention; they have even been reinterpretated as connective tissue. From our own observations on living animals, we are now able to give a more precise description of these muscular tracts.

Each bundle consists of 30 to 50 muscle fibers, tightly packed into a flat ribbon contained in the sagittal plane; the bundles follow one edge of the pharynx wall along its entire length. At their aboral extremities, all the muscle fibers in each bundle are attached to the walls of the axial aboral canal which is a continuation of the pharynx cavity immediately below the apical sensory organ (see Hyman, 1940). Muscle fibers

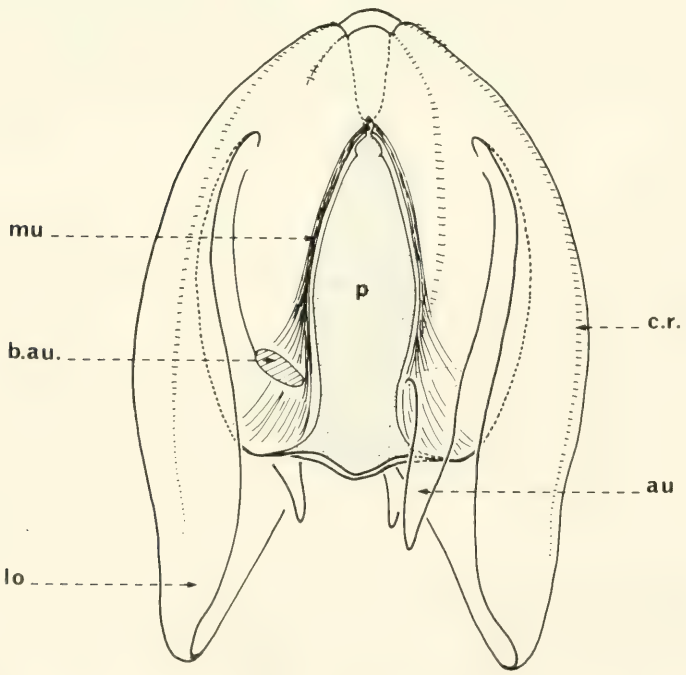


FIGURE 2. Diagram of *Mnemiopsis* drawn after Figure 1 showing the course of the sagittal muscle bundles (mu) along the pharynx (dotted). At their oral extremities, the muscle fibers may either enter the base of the auricle (au) or lips; same legends as in Figure 1. On the left part of the drawing the auricle has been cut away (b.au; cut base of the auricle), and the paratransversal comb row is not represented.

progressively spread out from the bundle and insert on the body wall at various levels from the bases of the auricles down to the lips (Fig. 2).

Each muscle fiber appears as a transparent cylinder running through the entire length of the bundle. The length of the bundle—and thus of the fibers—is directly related to the size (and age) of the animal. The longest fibers are also the thickest. Our electron microscope studies were conducted on a sample of bundles ranging from 1 to 2.5 cm in length, whereas in experiments and fixations on single cells we used the longest bundles, up to 3.5–4 cm.

Light microscope observations on living cells

Undisturbed isolated cells, or cells kept in Ca-free ASW, appear as smooth surfaced cylinders, as in the intact animal. In our experiments, the extremities of single cells either taper into thread-like endings, or swell into a bulbous stump suggestive of resealing which has been demonstrated in *Beroe* muscle cells (Hernandez-Nicaise *et al.*, 1980) (Figs. 3, 4).

Upon weak mechanical or electrical stimulation, the cells contract and become coiled (Fig. 4). The coiling is either clockwise, counter-clockwise, or both, along a given cell. Usually a weak stimulus initiates a localized thickened coil which may spread slowly along the cell, so that part of the whole length of the cell will take this spring-like profile. Such coiled fibers are never observed in an intact animal nor in bundles.

Further stimulation or excess external K^+ (100 m*M*) induces a strong contraction leading to a full shortening of the fiber. Usually it undergoes a brief coiled phase, then shortens dramatically into a thick straight cylinder with a transversally plicated surface (Fig. 5). Such an extreme shortening cannot be obtained *in situ:* in the intact mesoglea a strongly stimulated fiber will break into a row of bead-like fragments. In most cases an isolated cell will not stand such extreme shortening: the membrane usually bursts apart at one end, or the contractile cytoplasm detaches from the cell membrane and recoils at one extremity of the cell.

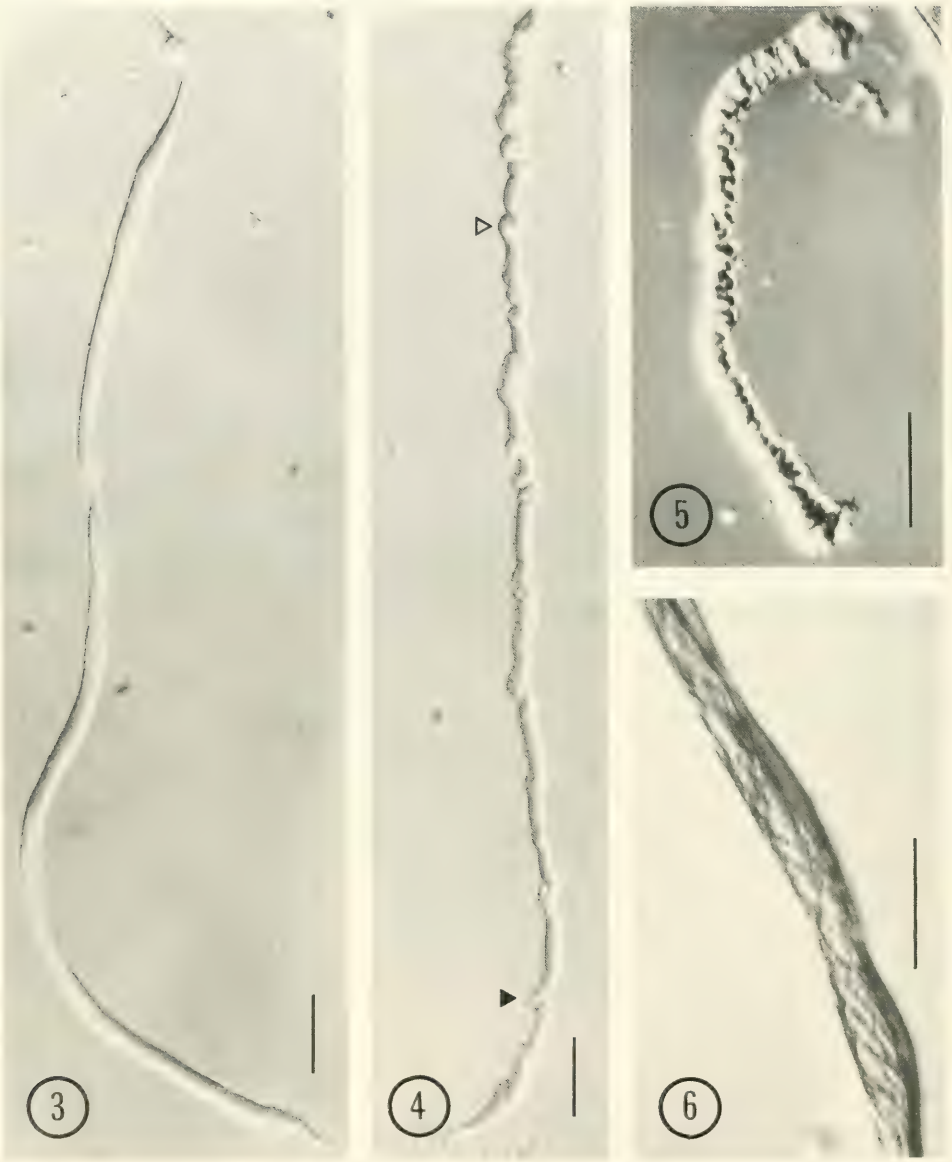
Under Nomarski optics, the fibers exhibit an axial row of nuclei with no intervening septa. The muscle fibers of *Mnemiopsis* thus appear as long multinucleated cells. The contractile cytoplasm shows no transverse or oblique striation. It displays a fibrillar organization: fibrils run along the entire length of the cell and are entwined in crossed helices (Fig. 6).

Electron microscope study

The ultrastructure of cells *in situ* and of single cells appears similar. No modifications suggestive of damage caused by the enzymatic dissociation have been found in isolated cells. The following description is thus largely based on a survey of several bundles, with additional data gained from the study of sections of isolated cells.

Examination of cross sections of various bundles confirms that the diameter of muscle cells is correlated with their length, and shows that the cells of one bundle are fairly homogenous in size. The shortest bundle examined has the smallest fibers, from 3 to 4.3 μm in diameter (average: 3.5 μm, 30 fibers), and the longest bundle has the largest fibers, with diameters up to 36 μm (Figs. 7, 8).

The muscle cells of a bundle are attached to their neighbors by thin strands of dense mesoglea, usually less than 1 μm in width (Fig. 7). Each cell is surrounded by a basal lamina, probably modified by the hyaluronidase treatment, but nevertheless densely stained by tannic acid (Figs. 7, 8, 10).



FIGURES 3–6. Micrographs of living muscle fibers isolated from the longitudinal sagittal bundles of *Mnemiopsis;* Nomarski interference contrast.

FIGURE 3. Relaxed muscle fiber. The upper end has probably been cut during isolation and is slightly bulbous. Note the smooth sarcolemmal surface and the axial row of organelles. Scale bar: 100 μm (100×).

FIGURE 4. Coiled muscle fiber. Parts of the fiber coil clockwise (dark triangle) while others coil counterclockwise (open triangle). Note again the bulbous stump at one extremity. Scale bar: 100 μm (100×).

FIGURE 5. Fully contracted fiber. The cell has the appearance of a tightly compressed spring. The contraction has been obtained by exposing the cell to 100 m*M* external K^+. Scale bar: 100 μm (150×).

FIGURE 6. Relaxed cell, slightly compressed to show the double spiral pattern of fibrils. Scale bar: 50 μm (350×).

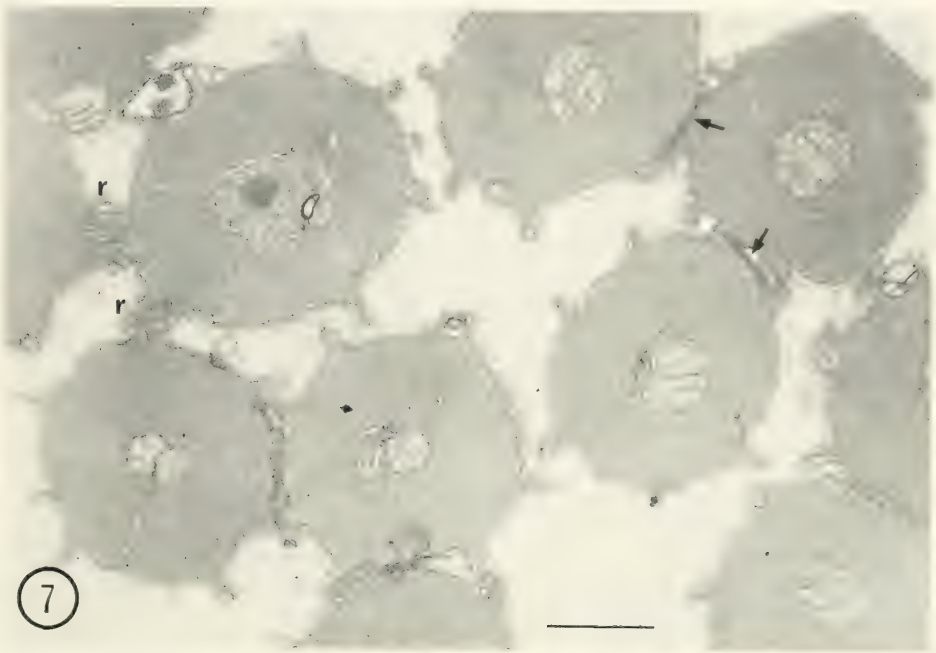


FIGURE 7. Part of a sagittal muscle bundle seen in cross section from a small *Mnemiopsis*. The diameter of the cells appears uniform. The cells are mechanically coupled by their longitudinal ridges (r) and/or strands of thickened mesoglea (arrows). Scale bar: 2 μm (7000×).

As observed with light microscopy the axial core contains a row of nuclei. The nuclei are euchromatic with a single large nucleolus, and have a smooth oval outline in relaxed cells (Figs. 7, 8, 11). They are surrounded by various organelles: sacs and tubules of rough and smooth endoplasmic reticulum, small Golgi bodies with their associated vesicles, and mitochondria. Between these organelles the axial cytoplasm may be filled by polysomes and free ribosomes, or by bundles of myofilaments.

Mitochondria are restricted to the central core. Their number increases with the size of the muscle fiber. In our preparations they display a clear matrix which sometimes seems swollen, a feature known to occur in other ctenophores (Hernandez-Nicaise and Amsellem, 1980). If the cells have been allowed to remain in ASW containing Ca^{++} (10 m*M*) the mitochondrial matrix contains several dark granules similar to those described in *Beroe* muscles (Nicaise and Hernandez-Nicaise, 1980).

A peculiar feature of these organelles is the continuity of their external membrane with the outer nuclear envelope. Such contacts have been regularly observed in various sections from different animals.

Myofilaments. In all cells examined, two types of filaments have been found, thick and thin (Figs. 8, 9, 10). Their size and distribution have been studied in cross sections of cells from bundles incubated in Ca-free ASW prior to fixation, these cells are thus presumed to be in a relaxed state.

The thick, myosin-like filaments have a minimum diameter of 15.30 nm ± 1.79 (S.D.). Their density is 249 ± 17 (S.D.) filaments per μm^2 of organelle-free contractile cytoplasm in cross-section, and is remarkably constant in the sample studied, varying within a small range from cell to cell (231–264 filaments/μm^2) and within each cell (less than 10% variation). These filaments are regularly distributed in a nearly hexagonal

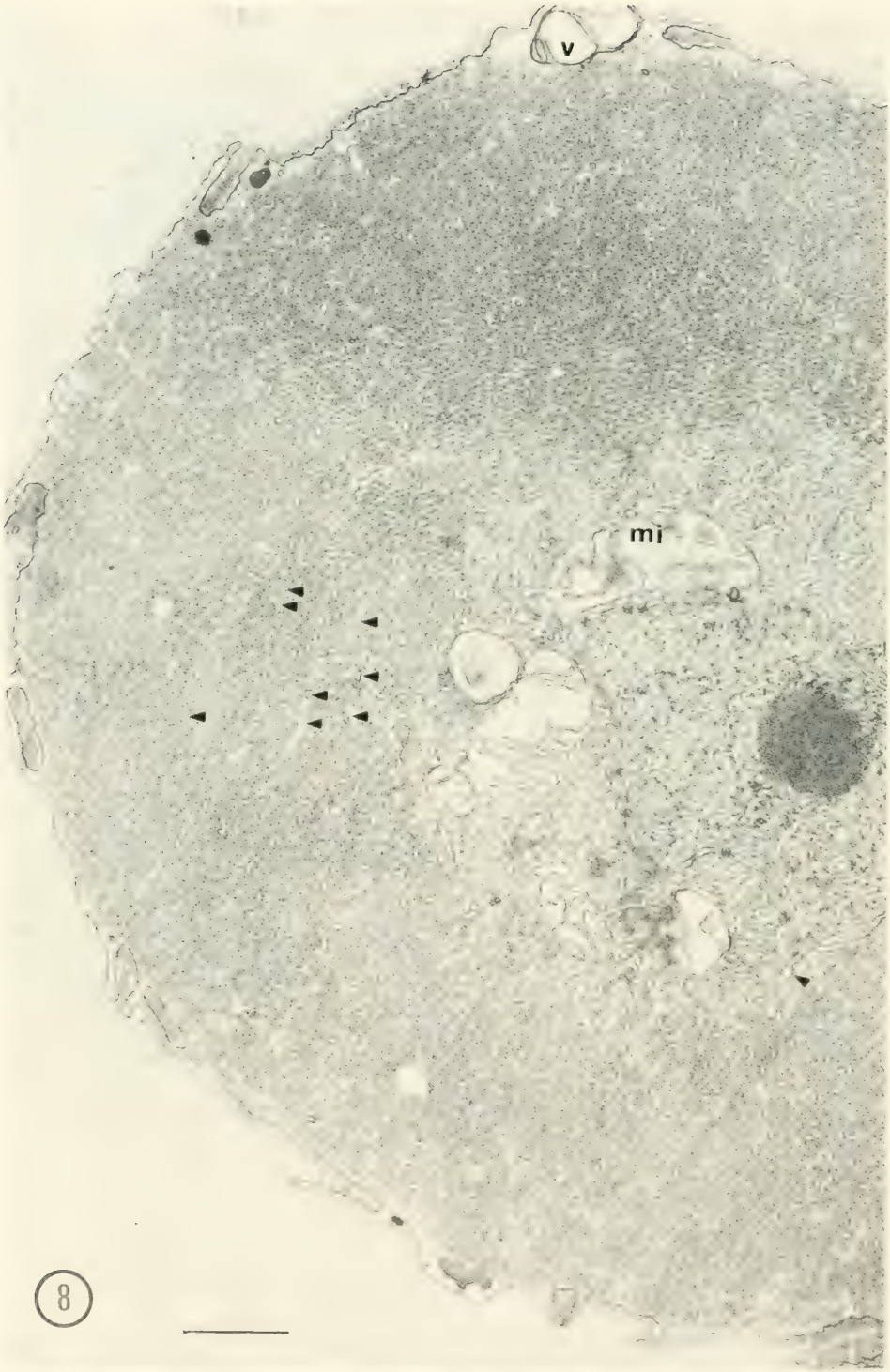
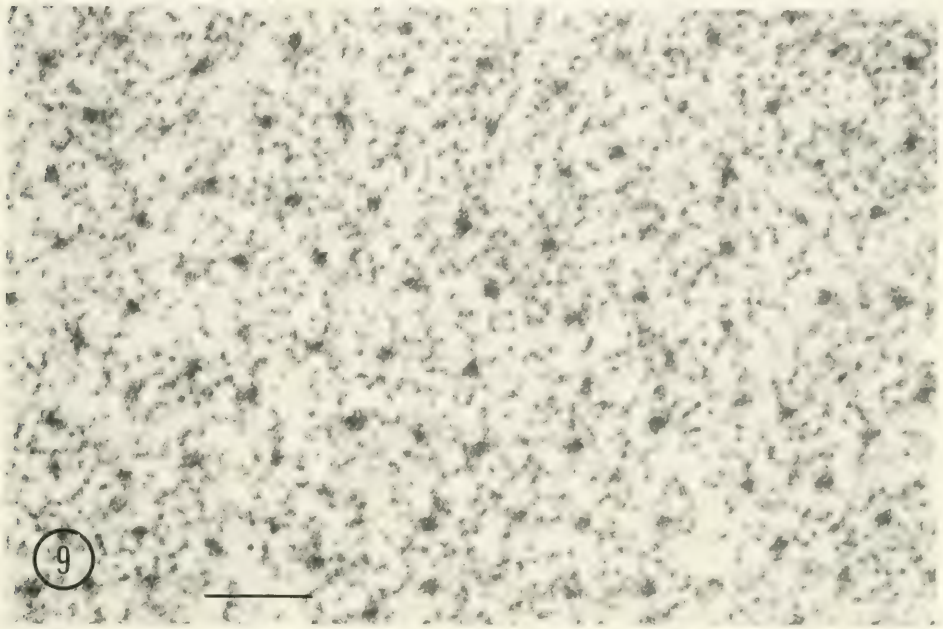


FIGURE 8. Cross section of an *in situ* muscle fiber from a medium sized *Mnemiopsis*. The axial nucleus is surrounded by several mitochondria (mi). The myofilaments are cut at various angles, the overall pattern suggesting an helicoidal arrangement. Note the numerous small SR tubules (arrowheads), nearly all sectioned transversally, and the clear "vesicles" tightly apposed to the cell membrane. Scale bar: 1 μm (14,500×).

pattern (centered hexagons), with a center-to-center spacing varying from 70 to 95 nm (Fig. 9).

The thin, actin-like filaments have a diameter of 5.9 ± 0.84 nm. If the bundles are incubated in a solution containing the S1 fragment of myosin (from chick smooth muscle), the thin filaments exhibit the classical arrowhead configuration. On this basis



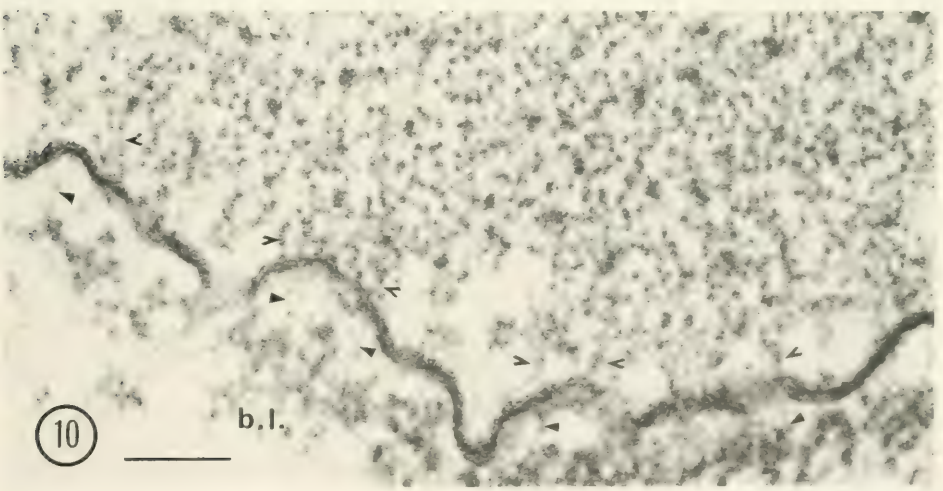


FIGURE 9. Transverse section of thick and thin myofilaments. The thick, myosin-like filaments form a nearly hexagonal lattice. The thin actin filaments form irregular rosettes around the thick myofilaments and are linked by finer microfibrils. Scale bar: 0.1 μm (140,000×).

FIGURE 10. Periphery of a muscle fiber in cross section. Microfibrils link the myofilaments and attach on the plasmalema (open arrows). At the same sites fibrils originating from the basal lamina (b.l.) insert on the external side of the cell membrane (arrowheads). Scale bar: 0.1 μm (140,000×).

they will be referred to here as actin filaments. The ratio of thin-to-thick filaments is very stable, varying from 7.2 to 7.3, with a mean value of 7.22.

In some areas of cross sections the thick and thin filaments are linked by radial cross-bridges stemming from the thick filaments. There is no definite orbital pattern, the thin filaments being arranged in irregular rosettes around the thick filaments (Figs. 9, 10). In restricted patches between thick filaments, adjacent actin filaments are linked by strands of an amorphous electron-dense material building an irregular network.

No striation pattern or dense anchoring structures such as intracytoplasmic dense bodies or peripheral attachment plates have been found in these muscle fibers. However, following tannic acid block staining, discrete patches of fuzzy material can be seen underlying the inner leaflet of the cell membrane. This internal coat is continuous with the material linking the actin filaments. At these same localizations, microfilaments, originating from the basal lamina, attach to the external leaflet of the cell membrane (Fig. 10).

In longitudinal sections of relaxed isolated cells, the myofilaments run nearly parallel to the axis of the fiber and there is no evidence of existing myofibrils. In cross or longitudinal sections of coiled fibers, the overall aspect of the contractile cytoplasm is a zig-zag pattern. The myofilaments appear grouped in bundles cut at various angles, and delineated by a narrow clear space containing a tubule of the SR or one or several microtubules. In a given section the width of such bundles varies from 0.6 to 2 μm (a more accurate estimate requires serial sectioning). We believe that these bundles are the myofibrils observed in living cells.

Surface of muscle cells. Neither cells observed in sections from bundles nor isolated cells exhibit any permanent system of sarcolemmal invaginations. Some occasional infoldings may occur and are easily recognized in tannic acid-contrasted tissues.

In the bundles so far examined, the surface of muscle cells shows longitudinal ridges which appear in cross sections as short (less than 1 μm) finger-like evaginations. The distribution of these ridges very nearly follows an axial symmetry (see Figs. 7, 8).

Single cells do not display these ordered ridges, but instead show numerous slender processes, which are mobile in living cells (Fig. 11).

Sarcoplasmic reticulum. In all the sections observed—from *in situ* and isolated cells—the SR appears distributed among the whole contractile cytoplasm. It is distinctly impregnated by tannic acid in isolated cells which have been fixed immediately upon enzymatic dissociation with no further recovery in normal ASW. From such preparations, the SR appears as a set of longitudinal tubules running along the myofilaments; they may be linked by lateral branches (Figs. 11, 12) or merge into larger vesicles. In longitudinal sections intersecting the nuclei, the tubules are in continuity with the nuclear envelope.

The SR is not particularly abundant in the immediate vicinity of the plasma membrane. No peripheral coupling has been found so far in *Mnemiopsis* fibers, although some vesicles are very close to the cell membrane.

On cross sections the SR profiles are irregularly distributed among the contractile cytoplasm, but their distribution is not random: in most cases the SR sections are aligned on imaginary spirals originating in the axial core toward the periphery of the cell (see Figs. 7, 8).

From our sample of bundles the following quantitative data have been obtained:

The SR profile in cross-section has an average area of 7.6×10^{-3} μm^2 ($\pm 0.3 \times 10^{-3}$ SEM) and an average perimeter of 0.36 μm ($\pm 8.5 \times 10^{-3}$). The SR accounts for 0.91% (± 0.035) of the volume of the contractile cytoplasm, and its relative surface

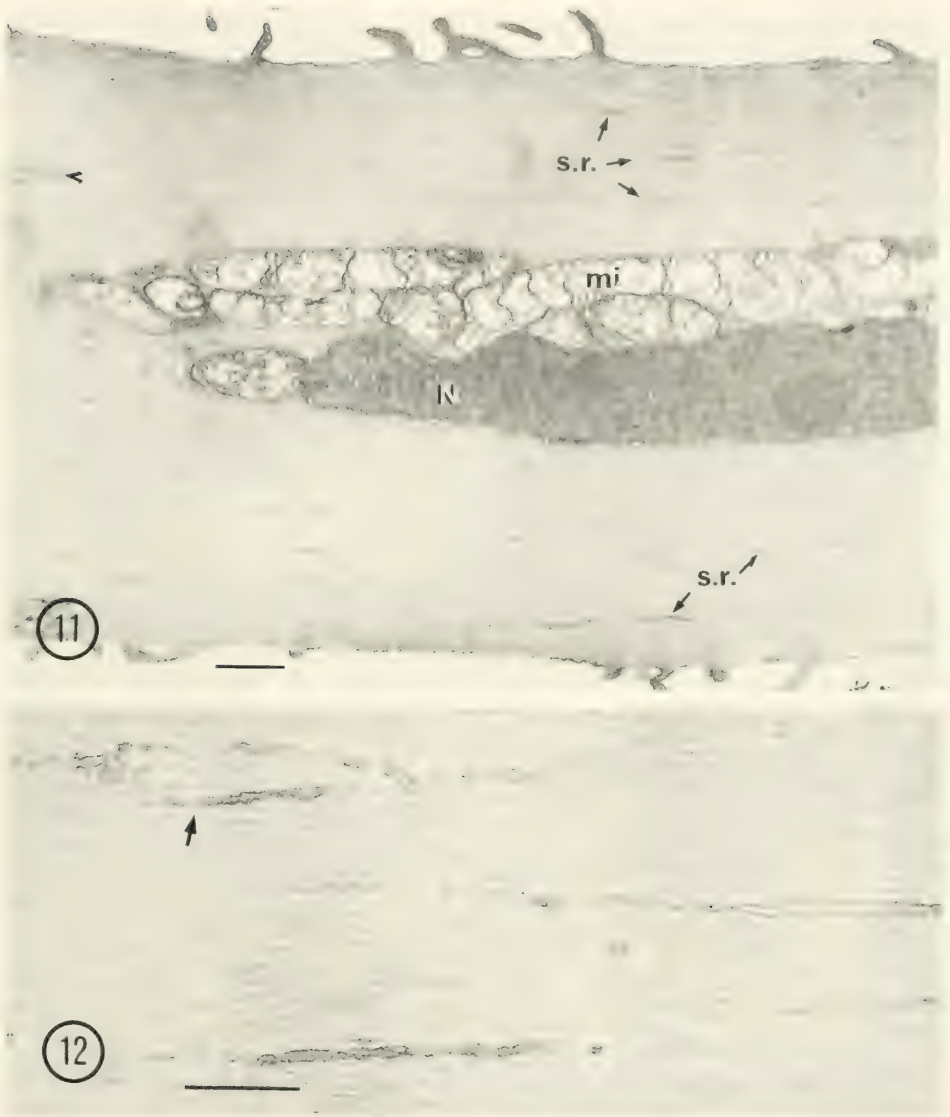


FIGURE 11. Longitudinal section of a relaxed isolated muscle fiber through the axial core. The elongated nucleus (N) is flanked by tightly packed mitochondria (mi). The SR (s.r.) is densely stained by tannic acid. A branching of a tubule is visible at the upper left (open arrow). Scale bar: 1 μm (9000×).

FIGURE 12. Detail of the SR tubules from another section of the same cell as in Figure 11. The arrow points to a "mesh" of the sarcotubular network. Scale bar: 0.5 μm (30,000×).

is 0.45 $\mu m^2/\mu m^3$ ($\pm$0.018) if we assume a complete consistency with Weibel's parallel cylinders model (1972). It may amount to 0.57 $\mu m^2/\mu m^3$ ($\pm$0.023) if we assume that the organization of the SR elements is completely isotropic amongst the myofilaments (see Discussion). The numerical density of SR profiles in the contractile cytoplasm is 1.26 tubules per μm^2 ($\pm$0.061). These values are homogeneous for the whole sample despite the large variability in size of the muscle cells (one way variance analysis, $F = 1.40$).

There is a slight but significant correlation between the value of the ratio Asr/Acc (or Bsr/Acc) and the area of contractile cytoplasm of the section if they are calculated on each section ($r = 0.35$ in both cases, $P < 0.5\%$).

Junctions. We looked for intermuscular junctions in all bundles studied. Few were found with most of them located between fibers of the periphery of the bundles.

At these junctions the plasma membranes are apposed along variable lengths (0.3–4 μm), and may form close contacts at some restricted patches. At these close contacts, tannic acid post osmium stains the intercellular space—4 to 5 nm wide—

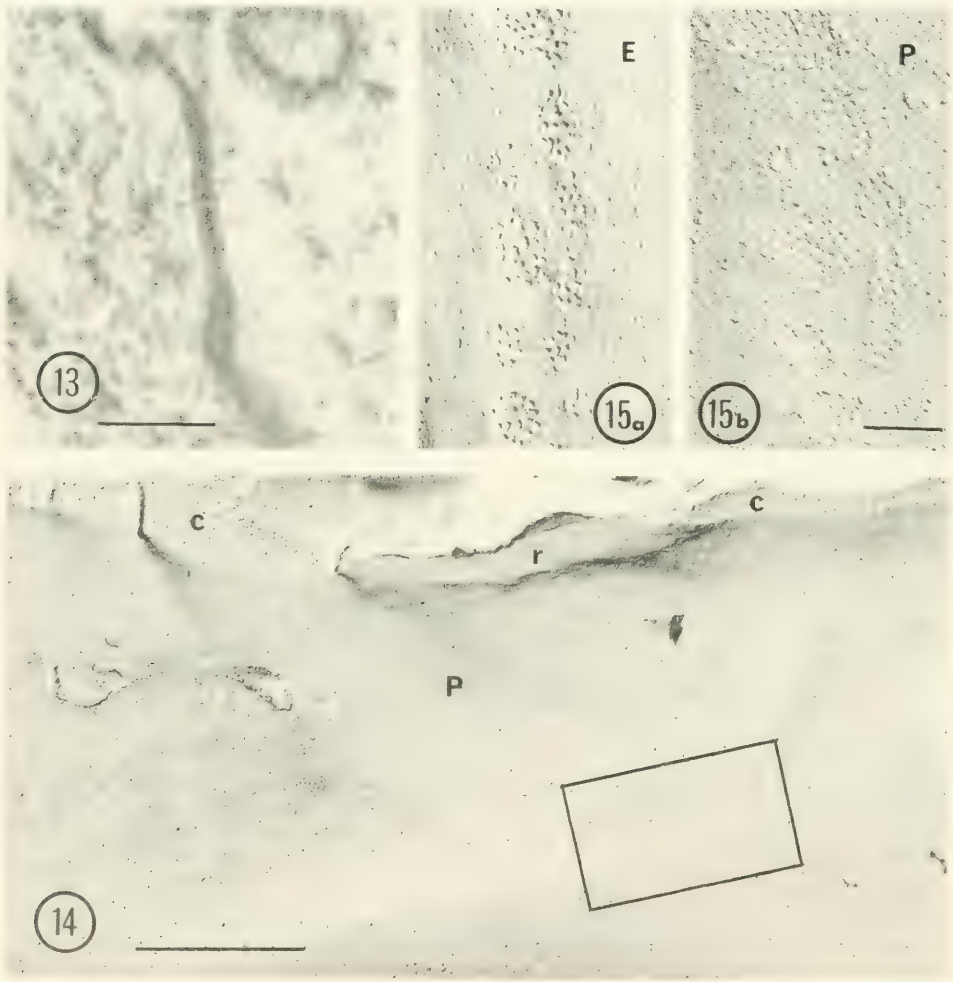


FIGURE 13. Junction between two cells of the bundle. Gap is stained giving rise to a pentalayered structure (tannic acid 1% post osmium). Note the thin bridges across the gap. Scale bar: 0.1 μm (150,000×).

FIGURES 14–15a, b. Freeze-fracture replicas of a muscle fiber.

FIGURE 14. The cleavage plan exposes the P face of the cell membrane and runs through a longitudinal ridge (r) thus exposing a band of cytoplasm (c). The replica shows a large array of polygonal gap junctional plaques, intercalated with particle-free aisles. Scale bar: 0.5 μm (44,000×).

FIGURES 15a, b. Complementary freeze-fracture replicas of part of the junction of Figure 14. E-face particles (Fig. 15a) correspond to a honeycomb array of pits on the P-face (Fig. 15b) (framed area in Fig. 14). Scale bar: 0.1 μm (100,000×).

thus revealing a pentalayered structure (Fig. 13). The intercellular space contains bridges repeating every 16–20 nm.

In freeze-fracture replicas, features characteristic of gap junctions were found on some muscles (Figs. 14, 15a, b). The E-face, or ectoplasmic fracture, of the muscle membrane exhibits polygonal clusters of 11–14 nm particles (Fig. 15a) and a complementary ordered lattice of pits—with a 17 nm center-to-center spacing—on the P, or protoplasmic fracture, face (Figs. 14, 15b). The clusters may be isolated or appear in large arrays (Fig. 14).

In any cross section of a bundle, nearly each muscle profile is in contact with one or two clear circular profiles (0.3–1 μm in diameter) encased between two projections of the muscle membrane. These vesicles do not contain any structure except for an occasional network of microfilaments. At some discrete points the muscle cell and the "clear vesicle" come into close contact, with an intercellular space of 20–40 nm. Survey of the mesoglea surrounding the bundle has not given evidence for a special category of cell as the origin of these structures. We have found that muscle cells of the bundle and of the neighboring mesoglea can emit "blisters" of variable sizes which are apparently empty. We have not observed such evaginations in contact with a neighboring muscle cell. In some cases these "blisters" make reflexive contact with the mother cell. Some of the gap junctions observed in our freeze-fracture replicas may thus fall into this category of junctions.

Discussion

Viability of isolated cells

Our light and electron microscope observations together with the electrophysiological data reported previously (Hernandez-Nicaise *et al.*, 1981) demonstrate that enzymatically isolated muscle cells of *Mnemiopsis leydii* are functional cells: (1) the membrane appears ultrastructurally and physiologically intact, (2) the cells contract if stimulated or in the presence of excess of K^+ ions in the bathing fluid, and (3) the ultrastructural features appear unchanged.

Myofilaments

We consider that the thick and thin filaments are myosin and actin filaments, respectively. As already mentioned the thin filaments are decorated by the S1 subfragment of heavy meromyosin, and preliminary data from gel electrophoresis indicate that *Beroe* muscle cells contain myosin but not paramyosin (A. V. Somlyo, pers. comm.).

In ultrastructural cross sections the overall pattern of actin and myosin is very similar to the distribution observed in *Beroe* muscle cells (Hernandez-Nicaise and Amsellem, 1980). However, the quantitative data for myosin density and actin-to-myosin ratio differ significantly between the two species as reviewed in Table I.

The actin-to-myosin ratio is distinctly lower than the values given for vertebrate smooth muscle (see review in Somlyo, 1980) and is closer to the figures published for several invertebrate phyla (see review in Hernandez-Nicaise and Amsellem, 1980; Plesch, 1977).

Organization of contractile units

One of the most striking features of *Mnemiopsis* muscle cells, apart from their length, is the absence of peripheral and intracytoplasmic dense bodies.

TABLE I

Comparison between morphological parameters of Mnemiopsis leydii[a] *and* Beroe ovata[b] *muscle filaments*

	Mnemiopsis leydii in situ cells relaxed	*Beroe ovata in situ* cells state unknown	*Beroe ovata* isolated cells relaxed	*Beroe ovata* isolated cells coiled
Thick filament diameter	15.30 ± 1.79 nm	16.4 ± 0.3 nm	16.11 ± 2.12 nm	16.11 ± 2.12 nm
Thick filament density	249 ± 17 fil./μm^2	320–450 fil./μm^2	457 ± 15 fil./μm^2	350–660 fil./μm^2
Thick filaments spacing	70–95 nm	40–60 nm		
Thin filament diameter	5.9 ± 0.84 nm	6.2 ± 1.6 nm	6.3 ± 0.6 nm	6.3 ± 0.6 nm
Thin/thick filament ratio	7.22	2.5–7.5	5.22 ± 0.21	4.88 ± 0.29

[a] Means ± S.D.

[b] Data from Hernandez-Nicaise and Amsellem (1980) and Hernandez-Nicaise *et al.* (1982).

In vertebrate smooth muscles the dense bodies are the morphological and functional correlates of fragmented Z lines (Bond and Somlyo, 1982). Their spatial distribution in oblique strands across the cell points to an organization of the myofilaments into small contractile units (Bond and Somlyo, 1982; Fay *et al.*, 1983). A similar type of organization is found in invertebrate smooth muscles like the byssus retractor of *Mytilus* (Sobieszek, 1973). From our observations reported in the present paper, we propose that, as in *Beroe* (Hernandez-Nicaise *et al.*, 1982), the myofilaments may be organized into two sets of thin myofibrils attached at their extremities on the muscle membrane along two (or two sets of) enantiomorphic helices.

The small patches of sarcolemma coated internally by a filamentous coat are likely candidates as mini-attachment plates. The microfilaments linking those patches to the basal membrane are suggestive of fibronectin microfilaments known in other cell types to "cooperate" with actin bundles (Singer, 1979; and review by Hynes, 1981). Such bonds between the muscle membrane and the mesoglea may explain the fragmentation of muscle fibers under the action of fixatives.

Each of the oblique myofibrils may be constituted of serial units, *i.e.*, pseudosarcomeres. The diameter of the muscle fibers and the low angle between the myofilaments and the fiber axis at rest, call for very long fibrils; but the possibility of a 75% shortening of single fibers implies that a fibril is made of a series of units with an important overlapping of myosin and actin filaments, rather than a single pseudosarcomere with one set of myosin filaments. The staggering of myosin and actin filaments in such small myofibrils accentuated by an unavoidable shearing, may thus account for the fact that in cross sections actin and myosin filaments are distributed as in A-bands of obliquely striated muscles fixed in a contracted state (see review in Rosenbluth, 1972).

The structural and biochemical equivalents of intracytoplasmic dense bodies therefore have to be demonstrated in order to establish the existence of serial pseudosarcomeres. Indications of such attachment structures have already been obtained from electron microscopy of highly stretched *Beroe* muscles (unpub. obs.).

Z elements with little or no contrast have been shown (if not specifically reported) in other primitive metazoans, namely Cnidaria. In figures published by various authors, we have noted that in cnidarian epitheliomuscular cells, the smooth muscle fibrils are devoid of dense bodies, and that striated myofibrils display a thin, wavy strand of fuzzy material in place of Z lines (Chapman, 1974; Amerongen and Peteya, 1976; Keough and Summers, 1976; Anderson and Schwab, 1981). Hoyle (1983) reports a striated muscle in a primitive Crustacean with "barely discernible Z regions."

Mnemiopsis muscles (and all other ctenophore muscles we have observed so far) also lack the so-called intermediate filaments, or desmin filaments. From published figures these filaments are equally absent in Cnidaria muscles. Desmin filaments occur in both smooth and striated muscles, and are associated with dense bodies (Small and Sobieszek, 1977; Bond and Somlyo, 1982) and the Z line (Behrend, 1977; Lazarides, 1980), respectively. The protein α-actinin is one of the main components of the Z line and dense bodies (Ebashi *et al.*, 1966; Schollmayer *et al.*, 1976; Endo and Nasaki, 1982) and is responsible for most of the electron-opacity of these structures together with desmin and vinculin (see review in Lazarides, 1980). Ctenophores and Cnidaria may lack α-actinin and desmin and may use other proteins (see review in Weeds, 1982), with a weak affinity for electron microscope stains, as bonds between the actin filaments and anchoring structures.

Sarcoplasmic reticulum

The SR of the giant muscle cells of *Mnemiopsis* is very similar in its morphology and distribution to that of *Beroe* giant muscle cells (Hernandez-Nicaise and Amsellem, 1980; Malaval *et al.*, 1981; Malaval, 1982). In both species the heterogeneous distribution of SR in the myoplasm and the alignment of SR profiles in cross sections along spirals, suggest an ordered three-dimensional pattern of this organelle along the fiber, which may reflect to some extent the organization of the myofibrils (Hernandez-Nicaise *et al.*, 1982; Malaval, 1982).

Our estimates of SR relative volume and correlated parameters have been calculated on the assumption that the SR distribution in relaxed *Mnemiopsis* muscles follows Weibel's model of parallel tubules (1972). Table II gives the values obtained for *Mnemiopsis* and *Beroe* muscles. It is theoretically possible to assess the relevance of this model for a given specimen by comparing the values obtained for Sv in different planes of section (Eisenberg *et al.*, 1974). However, such a control appears only feasible on striated fibers, for which an estimate of the angle between the plane of section and the axis of the cell can be calculated from the length of sarcomeres. With *Mnemiopsis* fibers we can only consider the extreme values of Sv, the "real" value probably being closer to the figure obtained with Weibel's anisotropic model.

To summarize, the relative volumes of SR in *Mnemiopsis* and *Beroe* muscles are closely similar in value but in *Mnemiopsis* the tubules are smaller and more numerous per cross sectional unit.

TABLE II

Comparison between morphological parameters of SR in muscle fibers of Mnemiopsis leydii[a] *and* Beroe ovata[b]

		Average area of SR tubule in cross section	Average circumference of SR tubule in cross section	Average diameter of SR tubule in cross section	Relative volume of SR in % of contractile cytoplasm	Surface density of SR, expressed in $\mu m^2/\mu m^3$	Numerical density of SR tubules in cross sections
Mnemiopsis		$7.6 \cdot 10^{-3}\ \mu m^2 \pm 0.3 \cdot 10^{-3}$	$0.36\ \mu m \pm 8.5 \cdot 10^{-3}$	$0.114\ \mu m$	$0.91\% \pm 0.035$	0.45 ± 0.018	1.26 ± 0.026
Beroe	1	$15 \cdot 10^{-3}\ \mu m^2 \pm 1 \cdot 10^{-3}$	$0.48\ \mu m \pm 20 \cdot 10^{-3}$	$0.152\ \mu m$	$1.24\% \pm 0.070$	0.40 ± 0.020	0.90 ± 0.070
	2	$19 \cdot 10^{-3}\ \mu m^2 \pm 2 \cdot 10^{-3}$	$0.59\ \mu m \pm 30 \cdot 10^{-3}$	$0.186\ \mu m$	$0.70\% \pm 0.50$	0.22 ± 0.020	0.38 ± 0.030

[a] Means ± S.D.

[b] Data from Malaval (1982). The values were obtained from two samples from two animals and the two sets of figures differed significantly and are thus given separately (see Schmalbruch, 1979, for a similar problem with muscle parameter variability in a vertebrate).

The wide range of sizes in our sample of sagittal bundles of muscle cells in *Mnemiopsis* has enabled us to find a positive correlation between fiber size and the SR volume.

The relative volume of SR of ctenophore giant smooth muscles is thus low if compared with the figures generally given as reference for vertebrate smooth muscle, namely 5.75% in vascular smooth muscle and 2% in phasic smooth muscle (Somlyo, 1980). However very similar figures have been reported for mammalian smooth and striated muscles (Schmalbruch, 1979; McGuffee *et al.*, 1981). Furthermore, these studies show the unsuspected influence in such quantitative studies of various factors extrinsic to the preparation such as the fixation procedure (McGuffee *et al.*, 1981), the buffer used in fixing and rinsing solutions (Moriya and Miyazaki, 1979) and the physiological condition of the animal (Schmalbruch, 1979).

A relative volume of 1% is estimated by Somlyo (1978) as the minimum amount of SR to allow a release of Ca^{++} sufficient for activation of the contractile proteins. Assuming that this statement is true for ctenophores whose internal fluid and temperature differ widely from those of mammals, the value of 0.91% found in *Mnemiopsis* muscle cells is not too low to rule out that this organelle is involved in the onset of contraction.

Another important indication for the role of SR in contraction is the presence of peripheral couplings (or dyads) between sarcoplasmic cisternae and the plasma membrane which presumably are crucial in excitation-contraction coupling. No couplings have been observed in *Mnemiopsis,* and very few cisternae are found close to the membrane. In *Beroe* muscles most of these couplings are found at neuromuscular and intermuscular junctions. We may have missed them in *Mnemiopsis* as we were not specifically looking for neuromuscular junctions. This observation does not necessarily exclude the intervention of the SR in ctenophore muscles in excitation-contraction coupling and certainly does not rule out a role for this organelle in the relaxation of these giant fibers (Malaval *et al.*, 1981; Malaval, 1982).

If we adopt the criteria of classification proposed by Josephson (1975) and Plesch (1977), the giant muscles of *Mnemiopsis* and *Beroe* clearly fall into the same category. The muscle cells of the sagittal bundles of *Mnemiopsis* show fewer cross-bridges available per actin filament and fewer mitochondria than *Beroe* muscles. They are thus likely to be less powerful, less sustained, and slower than *Beroe* muscle cells. This is consistent with the behavioral data (Swanberg, 1974; Harbison *et al.*, 1978).

Junctions

The few intermuscular junctions observed in the sagittal bundles of *Mnemiopsis* present the features of gap junctions, and more precisely the ultrastructural characteristics of E-gap junctions which are predominantly found in invertebrates (see reviews in Flower, 1977; Peracchia, 1981; Larsen, 1983). The precise distribution of these junctions among the muscles of a bundle remains to be evaluated. In vertebrates, gap junctions are widely accepted as the ultrastructural correlate of electrical coupling (Peracchia, 1981). They occur between smooth muscle cells (Fry *et al.*, 1977) which work in a coordinated fashion; for example, in a pregnant uterus the number of gap junctions between myometrial cells increases significantly at the onset of labor (Garfield *et al.*, 1977).

The contacts described in this study may link together the fibers of the sagittal bundles. To date however, an electrical coupling between muscles of a bundle has not been found (Anderson, in press). The empty "blisters" point toward another possibility: they may be broken parts of radial muscle fibers destroyed during dissection. Such a possibility is also suggested by Anderson (in press).

With the isolation technique reported in this study, it is possible to use the giant

smooth muscle fibers of *Mnemiopsis* for a variety of studies while controlling them individually under a simple dissecting microscope. In addition, *Mnemiopsis* sagittal bundle of parallel fibers can be used as multicellular homogeneous sample for quantitative studies.

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LITERATURE CITED

AMERONGEN, H. M., AND D. J. PETEYA. 1976. The ultrastructure of the muscle system of *Stomphia coccinea*. Pp. 541–547 in *Coelenterate Ecology and Behavior*, G. O. Mackie, ed. Plenum Press, New York.

ANDERSON, P. A. V., AND W. E. SCHWAB. 1981. The organization and structure of nerve and muscle in the jellyfish *Cyanea capillata*. *J. Morphol.* **170:** 383–399.

BAGBY, R. M., A. M. YOUNG, R. S. DOTSON, B. A. FISHER, AND K. MCKINNON. 1971. Contraction of single smooth muscle cells from *Bufo marinus* stomach. *Nature* **234:** 351–352.

BAKER, L. D. S., AND M. R. REEVE. 1974. Laboratory culture of the lobate Ctenophore *Mnemiopsis mccradeyi* with notes on feeding and fecundity. *Mar. Biol.* **26:** 57–62.

BEHRENDT, H. 1977. Effect of anabolic steroids on rat heart muscle cells. *Cell Tissue Res.* **180:** 303–315.

BOND, M., AND A. V. SOMLYO. 1982. Dense bodies and actin polarity in vertebrate smooth muscle. *J. Cell Biol.* **95:** 403–413.

CAVANAUGH, G. M. 1956. *Formulae and Methods VI of the M.B.L. Chemical Room.* M.B.L., Woods Hole, Massachusetts.

CHAPMAN, D. M. 1974. Cnidarian histology. Pp. 2–36 in *Coelenterate Biology: Reviews and New Perspectives*, L. Muscatine and H. M. Lenhoff, eds. Academic Press, New York.

CHUN, C. 1880. Die Ctenophoren des Golfes von Neapels und der angrenzenden Meeres-Abschnitte, in *Fauna und Flora des Golfes von Neapel*, 4, Engelman, Leipzig.

CRUZ-ORIVE, L. M. 1980. Best linear unbiased estimators for stereology. *Biometrics* **36:** 595–605.

EBASHI, S., H. IWAKURA, H. NAKAJIMA, R. NAKAMURA, AND Y. OOI. 1966. New structural proteins from dog heart and chicken gizzard. *Biochem. Z.* **345:** 201–211.

EISENBERG, B. R., A. M. KUDA, AND J. B. PETER. 1974. Stereological analysis of mammalian skeletal muscle. *J. Cell Biol.* **60:** 732–754.

ENDO, T., AND T. MASAKI. 1982. Molecular properties and functions *in vitro* of chicken smooth-muscle α-actinin in comparison with those of striated muscle α-actinins. *J. Biochem.* **92:** 1457–1468.

FAY, F. S. 1976. Structural and functional features of isolated smooth muscle cells. Pp. 186–201 in *Cell Motility. A.*, R. Goldman, T. Pollard, and J. Rosenbaum, eds. Cold Spring Harbor Conf. on Cell Proliferation, Vol. 3, Cold Spring Harbor Lab., New York.

FAY, F. S., K. FUJIWARA, D. D. REES, AND K. E. FOGARTY. 1983. Distribution of α-actinin in single isolated smooth muscle cells. *J. Cell Biol.* **96:** 783–795.

FLOWER, N. E. 1977. Invertebrate gap junctions. *J. Cell Sci.* **25:** 163–172.

FRY, G. N., C. E. DEVINE, AND G. BURNSTOCK. 1977. Freeze-fracture studies of nexuses between smooth muscle cells. Close relationship to sarcoplasmic reticulum. *J. Cell Biol.* **72:** 26–34.

GARFIELD, R. S., S. SIMS, AND E. E. DANIEL. 1977. Gap junctions: their presence and necessity in myometrium during parturition. *Science.* **15:** 654–671.

HARBISON, G. R., L. P. MADIN, AND N. R. SWANBERG. 1978. On the natural history and distribution of oceanic ctenophores. *Deep Sea Res.* **25:** 233–256.

HERNANDEZ-NICAISE, M.-L., AND J. AMSELLEM. 1980. Ultrastructure of the giant smooth muscle fiber of the Ctenophore *Beroe ovata*. *J. Ultrastruct. Res.* **72:** 151–168.

HERNANDEZ-NICAISE, M.-L., A. BILBAUT, L. MALAVAL, AND G. NICAISE. 1982. Isolation of functional

giant smooth muscle cells from an invertebrate. Structural features of relaxed and contracted fibers. *Proc. Natl. Acad. Sci. U.S.A.* **79:** 1884–1888.

HERNANDEZ-NICAISE, M.-L., G. O. MACKIE, AND R. W. MEECH. 1980. Giant smooth muscle cells of *Beroe*. Ultrastructure, innervation, and electrical properties. *J. Gen. Physiol.* **75:** 79–105.

HERNANDEZ-NICAISE, M.-L., G. NICAISE, AND P. A. V. ANDERSON. 1981. Isolation of giant smooth muscle cells from the ctenophore *Mnemiopsis*. *Am. Zool.* **21:** 1012.

HOYLE, G. 1983. *Muscles and their Neural Control.* J. Wiley & Sons, New York. 689 pp.

HYMAN, L. H. 1940. *The Invertebrates: Protozoa through Ctenophora, Vol. I.* McGraw Hill, New York.

HYNES, R. O. 1981. Relationships between fibronectin and the cytoskeleton. Pp. 100–137 in *Cytoskeletal Elements and Plasma Membrane Organization,* G. Poste and G. L. Nicholson, eds. Elsevier/North Holland Biomedical Press, Amsterdam.

JOSEPHSON, R. K. 1975. Extensive and intensive factors determining the performance of striated muscle. *J. Exp. Zool.* **194:** 135–154.

KEOUGH, E. M., AND R. G. SUMMERS. 1976. An ultrastructural investigation of the striated subumbrellar musculature of the anthomedusan, *Pennaria tiarella*. *J. Morphol.* **149:** 507–526.

LAZARIDES, E. 1980. Intermediate filaments as mechanical integrators of cellular space. *Nature* **283:** 249–256.

LARSEN, W. J. 1983. Biological implications of gap junction structure, distribution and composition: a review. *Tissue Cell* **15:** 645–671.

MALAVAL, L. 1982. *Le reticulum sarcoplasmique des fibres musculaires lisses géantes de Beroe ovata (Cténaire). Etude morphologique, stéréologique et cytochimique.* Thèse de Troisième Cycle, N° 1149, Université Claude Bernard, Villeurbanne. 61 pp.

MALAVAL, L., G. NICAISE, AND M.-L. HERNANDEZ-NICAISE. 1981. Sarcoplasmic reticulum of the giant smooth muscle fiber of *Beroe*. *J. Gen. Physiol.* **78:** 22a.

MCGUFFEE, J., L. HURWITZ, S. A. LITTLE, AND B. E. SKIPPER. 1981. A ^{45}Ca autoradiographic and stereological study of freeze-dried smooth muscle of the guinea-pig vas-deferens. *J. Cell Biol.* **90:** 201–210.

MORIYA, M., AND E. MIYASAKI. 1979. Structural analysis of functionally different smooth muscles. *Cell Tissue Res.* **202:** 337–342.

NICAISE, G., AND M.-L. HERNANDEZ-NICAISE. 1980. Analytical electron microscopy of calcium sites in a giant smooth muscle cell: preliminary results. Pp. 483–488 in *X-Ray Optics and Microanalysis,* D. R. Beaman, R. E. Ogilvie and D. B. Wittry, eds. Pendell Publishing Co., Midland, Michigan.

PERACCHIA, C. 1981. Structural correlates of gap junction permeation. *Int. Rev. Cytol.* **66:** 81–146.

PLESCH, B. 1977. An ultrastructural study of the musculature of the pond snail *Lymnea stagnalis* (L.). *Cell Tissue Res.* **180:** 317–340.

ROSENBLUTH, J. 1972. Obliquely striated muscle. Pp. 389–420 in *The Structure and Function of Muscle,* G. H. Bourne, ed. Academic Press, New York.

SCHMALBRUCH, H. 1979. The membrane systems in different fibre types in the *triceps surae* muscle of cat. *Cell Tissue Res.* **204:** 187–200.

SCHOLLMEYER, R. E., L. T. FURCHT, D. E. GOLL, R. M. ROBSON, AND M. H. STROMER. 1976. Localization of contractile proteins in smooth muscle cells and in normal and transformed fibroblasts. Pp. 361–388 in *Cell Motility. A.,* R. Goldman, T. Pollard, and J. Rosenbaum, eds. Cold Spring Harbor Conf. on Cell Proliferation, Vol. 3, Cold Spring Harbor Lab., New York.

SINGER, I. I. 1979. The fibronexus: a transmembrane association of fibronectin-containing fibers and bundles of 5 nm microfilaments in hamster and human fibroblasts. *Cell* **16:** 675–685.

SMALL, J. V., AND A. SOBIESZEK. 1977. Studies on the function and composition of the 10-nm (100 Å) filaments of vertebrate smooth muscle. *J. Cell Sci.* **23:** 243–268.

SOBIESZEK, A. 1973. The fine structure of the contractile apparatus of the anterior byssus retractor of *Mytilus edulis*. *J. Ultrastruct.* **43:** 313–344.

SOMLYO, A. P. 1978. Role of organelles in regulating cytoplasmic calcium in vascular smooth muscle. Pp. 21–29 in *Mechanisms of Vasodilatation,* P. M. Vanhoutte, and I. Mleusen, eds. S. Karger AG, Basel.

SOMLYO, A. V. 1980. Ultrastructure of vascular smooth muscle. Pp. 33–67 in *Handbook of Physiology, Sect. 2, Vol. 2, Vascular smooth muscle,* D. F. Bohr, A. P. Somlyo, and H. U. Sparks, eds. Amer. Physiol. Soc., Bethesda, Maryland.

SWANBERG, N. 1974. The feeding behavior of *Beroe ovata*. *Mar. Biol.* **24:** 69–74.

WALSH, J. V. JR., AND J. J. SINGER. 1981. Voltage clamp of single freshly dissociated smooth muscle cells: current-voltage relationships for three currents. *Pflügers Archiv.* **390:** 207–210.

WARD, W. W. 1974. Aquarium systems for the maintenance of ctenophores and jellyfish, and for the hatching and harvesting of the brine shrimp (*Artemia salina*) larvae. *Ches. Sci.* **15:** 116–118.

WEEDS, A. 1982. Actin-binding proteins—regulators of cell architecture and motility. *Nature* **296:** 811–816.

WEIBEL, E. R. 1972. A stereological method for estimating volume and surface of sarcoplasmic reticulum. *J. Microsc.* **95:** 229–242.

Reference: *Biol Bull.* **167**: 229–237. (August, 1984)

INFLAMMATORY-LIKE REACTION IN THE TUNIC OF *CIONA INTESTINALIS* (TUNICATA). I. ENCAPSULATION AND TISSUE INJURY

NICOLÒ PARRINELLO, ELEONORA PATRICOLO, AND CALOGERO CANICATTÌ

Institute of Zoology, Palermo University, Via Archirafi, 18 90123 Palermo, Italy

ABSTRACT

Particulate (sheep erythrocytes, ascidian oocytes, stromata, colloidal carbon) or soluble agents (bovine serum albumin or hemoglobin, hemocyanin) were injected in varying doses into the tunic of *Ciona intestinalis*. This ascidian reacted by producing a capsule and/or tissue injury. Statistical analysis suggests that the two phenomena are independent, probably related to the nature and dose of the irritant.

Light histological observations showed granulocyte degranulation in the damaged tissue, suggesting that an acute inflammatory-like process is involved in the tunic reaction.

INTRODUCTION

To maintain body integrity, tunicates have evolved mechanisms which destroy and eject foreign materials. The defense responses include both humoral and cellular components. They could constitute a surveillance system ancestral to the vertebrate immune system (Parrinello and Patricolo, 1975, 1984; Parrinello *et al.*, 1977; Parrinello and Canicattì, 1982, 1983; Wright and Ermak, 1982).

Attempts to demonstrate immunological capabilities in the ascidian *Ciona intestinalis* have shown that it possesses natural (non-inducible) bacteriocidins (Johnson and Chapman, 1970) and hemagglutinins (Parrinello and Patricolo, 1975; Wright and Cooper, 1975) and that it reacts by phagocytosis and encapsulation to foreign materials inserted into the tunic (Parrinello *et al.*, 1977), and rejects a first set of tunic allografts (Reddy *et al.*, 1975). However the source of the natural defense responses needs further examination, while tunic graft rejection involves some persistent non-specific inflammatory responses.

C. intestinalis non-specifically reacts toward large concentrations of erythrocytes injected into the tunic and produces a capsule around them (Parrinello *et al.*, 1977). Cells infiltrate the area and release substances enveloping the foreign material. This response is often strong enough to produce large capsules visible through the tunic. Also in a variable number of treated specimens, the tunic matrix over the injected erythrocytes lysed, and a tunic wound was produced. Animals with the injured tunic survived for a period of time dependent on the seriousness of the trauma. In specimens which showed a slight reaction the wound healed. Preliminary light microscopic histological observations did not clarify the nature of such a reaction. Moreover, the data did not establish a relationship between the capsule and the injury.

In this study the *C. intestinalis* tunic injury and encapsulation produced by various doses of particulate or soluble agents were investigated by examining their external

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Abbreviations: SE = sheep erythrocytes; PBS = phosphate-buffered saline (0.01 *M* pH 7.4 phosphate buffer containing 0.15 *M* NaCl); BSA = bovine serum albumin; Hb = bovine hemoglobin; Hc = *Octopus vulgaris* hemocyanin.

appearance. It is shown that they are two un-related processes which most frequently appear as a result of high doses of either particulate material or soluble proteins. The capsule structure is described elsewhere (Parrinello and Patricolo, 1984). We now report some observations on the injured tissue. Histological studies relate the granulocyte degranulation to the injury process.

Materials and Methods

Adult *Ciona intestinalis* L. specimens (about 10–12 cm in length) were collected from the harbors of Palermo and Porticello, Italy. Animals showing a tunic free of external marine matter were selected; they were maintained in aerated and frequently renewed sea water at 15–18°C.

After several washings sheep erythrocytes (SE) (5×10^5, 5×10^6, 5×10^7 cells/ml) and colloidal carbon (G. Wagner, Lot C 11/1431 A) (2 and 20 mg/ml), were suspended in phosphate-buffered saline pH 7.4 (PBS). Hemoglobin-free red cell membranes were prepared using the method of Davis and Bakerman (1972); packed ghosts were suspended at final concentrations (v/v) of 1, 3, and 10% in PBS. The response to a more complex cellular system was investigated by injecting *C. intestinalis* oocytes collected from several specimens, washed and suspended in PBS ($1–1.2 \times 10^3$ oocytes/ml). Various concentrations (0.2, 2.0, and 20.0 mg/ml) of bovine serum albumin (BSA) (Sigma), bovine hemoglobin (Hb) (Sigma), and *Octopus vulgaris* hemocyanin (Hc) (kindly supplied by Dr. G. Nardi, Zoological Station, Naples), were prepared in PBS. 0.2 ml volumes of these preparations were injected into the tunic under the cuticle in the region of the sigmoid intestine with a syringe equipped with a 27-gauge needle.

Control specimens were injected with 0.2 ml PBS in each experiment.

The animals were inspected daily for signs of tissue reaction.

Statistical analyses were performed using analysis of variance; the acceptable level of significance was $P < 0.05$. For light optical studies a large fragment (about 1 cm wide) of the injured tunic was fixed in 70% ethanol and embedded in paraplast, 5 μm sections were stained with hematoxylin-eosin and Mallory's stains (Beccari and Mazzi, 1966).

Hemagglutinating activity was assayed as previously described (Parrinello and Patricolo, 1975). Blood was collected from the heart. Hemagglutination titers are expressed as reciprocal of the last dilution giving agglutination.

Results

The tunic reaction

Table I gives the external observations of the injected area during the tunic reaction. They show that, while PBS-injections never induce reaction in the control animals, particulate agents or soluble proteins can elicit two distinct types of tunic response. (1) SE, ascidian oocytes, BSA, Hb, and Hc induce a capsule which includes the injected materials. This appears 2 to 8 days after the injection as a whitish circular or elliptical disc (1.5–3.0 cm wide) included in the tunic tissue (Fig. 1). (2) SE, oocytes, the highest doses of stromata and colloidal carbon, BSA, and Hb induce a drastic response which produces a local injury of the tunic: a blister forms in the treated area, and the overlaying cuticle becomes thin and finally ruptures (Fig. 2a). When the foreign material is particulate the debris can disappear. In some specimens the two responses against SE, BSA, Hb, and Hc can occur together. In this case the injury can appear before or after the encapsulation. Both types of response show some degree

TABLE I

Reaction of Ciona intestinalis *to intratunic injection of particulate or soluble materials*

Irritant agent	Dose[1]	Injected specimens	Number of reacting specimens showing: Capsule A	Capsule plus injury B	Injury C	Days to produce 50% reacting specimens
SE	1×10^5	270	49 (18.1)	13 (4.8)	10 (3.7)	6–8
	1×10^6	422	176 (41.7)[a]	24 (5.7)	40 (9.5)	4–8
	1×10^7	937	332 (35.4)[b]	266 (28.4)[c]	33 (3.5)	3–6
Stromata from SE	1%	134	—	—	—	
	3%	111	—	—	—	
	10%	135	—	—	40 (29.6)	5
Oocytes	$1–1.2 \times 10^3$	50	36 (72.0)	—	12 (24.5)	5
Colloidal carbon	0.2 mg	113	—	—	—	
	2.0 mg	106	—	—	7 (6.6)	9
BSA	0.04 mg	100	1 (1.0)	19 (19.0)	—	8
	0.4 mg	100	16 (16.0)	40 (40.0)	—	4
	4.0 mg	100	—	8 (8.0)[c]	92 (92.0)	1
Hb	0.04 mg	130	4 (3.1)	—	—	4
	0.4 mg	124	48 (38.7)	15 (12.1)	2 (1.6)	2
	4.0 mg	126	14 (11.1)[d]	68 (53.9)[c]	14 (11.1)	2
Hc	0.2 mg	112	19 (16.9)	17 (15.2)	—	4
PBS	0.2 ml	627	—	—	—	

SE = sheep erythrocytes; BSA = bovine serum albumin; Hb = bovine hemoglobin; Hc = *Octopus vulgaris* hemocyanin; PBS = phosphate-buffered saline.

[1] In 0.2 ml. Percentage is reported in parentheses.

[a] $P < 0.01$ in comparison to B and C.

[b] $P < 0.05$ in comparison to C, $P < 0.01$ in comparison to C.

[c] $P < 0.01$ in comparison to C.

[d] $P < 0.01$ in comparison to B.

of modulation depending on the injurious agent. The capsule visible through the tunic appears in some of the specimens injected with erythrocytes (1×10^5–1×10^7), ascidian oocytes, BSA, Hb (0.04–4.0 mg), or Hc (0.4 mg). It is invisible when colloidal carbon or stromata is used even in high concentrations. The injury is produced by either particulate or soluble materials. Each agent, if injected in high doses, can elicit tunic damage as the only visible phenomenon (Table I).

The results in Table I suggest that encapsulation is not related to the injury formation. Significant differences ($P < 0.01$) were found between the frequencies of the capsule and injury responses produced by 1×10^6, 1×10^7 erythrocytes, or 4.0 mg BSA. Significant differences also resulted when "capsule plus injury" response was compared with each of the other two reactions produced when 1×10^6–1×10^7 SE, 4.0 mg BSA, or Hb were injected.

The dose of the irritant affects the response type and frequency, the largest dose being more effective in producing the tunic reaction. Different doses were examined statistically for each response type (Table II). Higher SE and protein concentrations

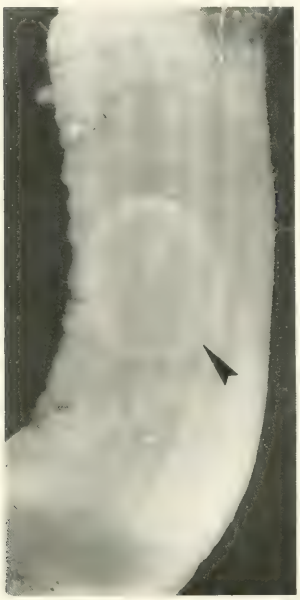

FIGURE 1. Capsule seven days after 0.4 mg bovine serum albumin injection into the tunic of *Ciona intestinalis* (arrowhead).

produced capsules in a significantly ($P < 0.01$) larger number of specimens. The highest doses also elicited an increased injury frequency.

There is variability in the time required for the appearance of the tunic reaction in about 50% of the reacting specimens in each treatment (Table I). The appearance time is inversely proportional to the dose and depends on the nature of the foreign material. Fast reactions (1–2 days) of either capsule, injury, or both were observed when concentrated protein solutions were used (4.0 mg BSA, 0.4–4.0 mg Hb) whereas slow reactions appearing in 4–8 days resulted at lower concentrations (0.04, 0.4 mg BSA, and 0.04 mg Hb). The same pattern characterized the effects of the various SE doses (see below) while only the highest concentration of stromata produced this response. The reaction time is also influenced by animal variability: in 10 different *C. intestinalis* groups (937 specimens) injected with the highest erythrocyte dose, about 50% of 631 specimens reacted within 3–6 days depending on each animal lot, the remainder reacted after 7–8 days. Variability in response frequency was also observed between the lots. The greatest variability characterized the mean frequency values of the injury responses. The mean values calculated from all experiments are indicated in Figure 3.

Hemagglutinating activity of the serum was tested with sheep or rabbit (RE) erythrocytes. To perform the assays the blood of five specimens was collected and pooled daily. Sera from PBS-injected animals showed no activity against SE, but agglutination titers of 2–4 were observed against RE. No changes were found in the hemagglutinating titers throughout the reaction period following SE injection.

The response toward particulate materials

Encapsulation is the most frequent response to erythrocytes and ascidian oocytes (Table I). Sheep erythrocytes can produce either a capsule or, less frequently, a tunic injury. Both responses are dose-dependent. The frequency of the tunic injury, even

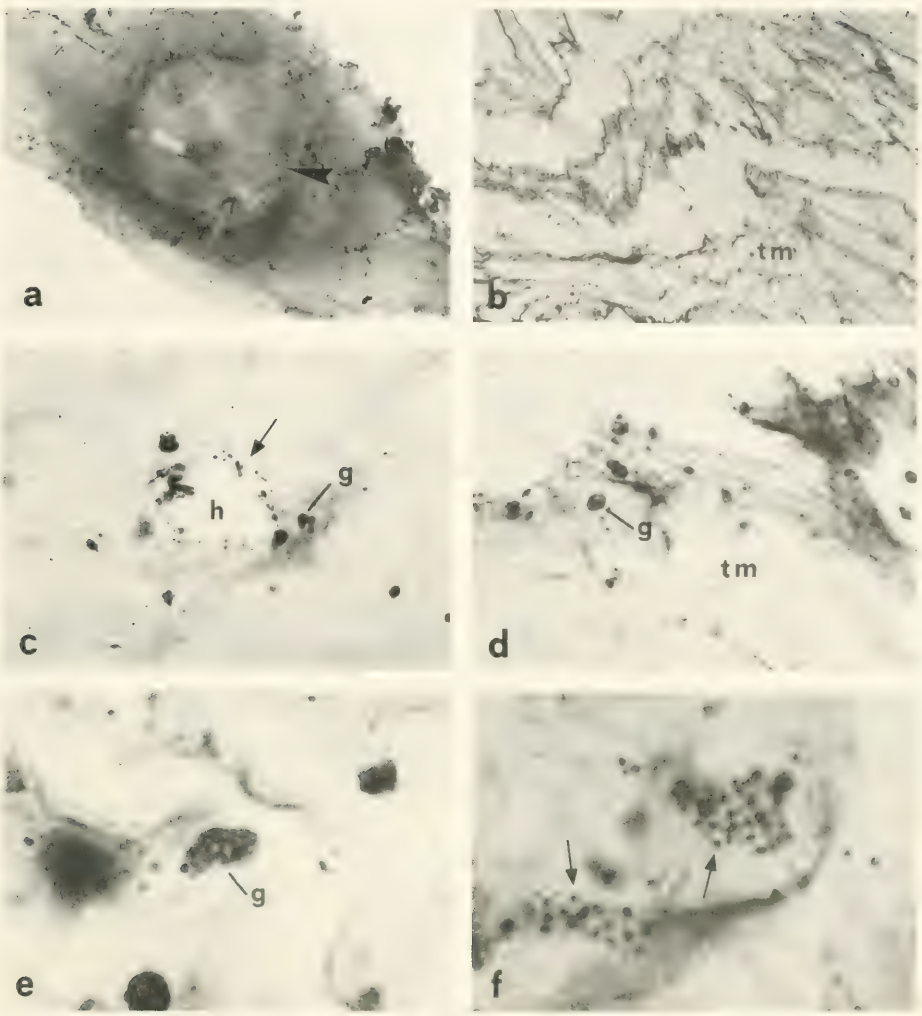

FIGURE 2. Tissue damage seven days after erythrocyte injection into the tunic of *Ciona intestinalis*. (a): Injured tunic (arrowhead); (b–f): transverse sections of the injured tunic (Mallory's stain); (b): lesion of the tunic, 80×; (c): hole in the tunic matrix (tm), 380×; (d): edge of the injured tunic, 380×; (e): eosinophil granulocyte, 1420×; (f): degranulation by cell membrane dissolution, 1420×. g = granulocyte; h = hole; arrows indicate granules.

when appearing with the capsule, increased with increased dosage (Fig. 3). The results in Figure 3 also show a pronounced variability in the responsiveness of the various animal groups to each dose.

The stromata at the highest dose and oocytes at the dose used also frequently elicited tunic injury, both being not very injurious agents. In fact, several days after the injection (5–8 days), these irritants produced tiny blisters (5–6 mm wide) containing gelatinous materials. Tissue damage rarely occurred when stromata were the irritants and the tunic subsequently healed.

Colloidal carbon was the least effective injurious agent even if injected in high quantities (2.0 mg/specimens). Only 6.6% of the 106 treated specimens showed a small blister like that described above.

TABLE II

Tunic reaction of Ciona intestinalis: *comparison of the dose response by analysis of variance*

Irritant agent	Dose compared	*P*-values[1]		
		Capsule	Capsule and injury	Injury
SE	1×10^5 vs. 1×10^6	$P < 0.01$	N.S.	$P < 0.01$
	1×10^5 vs. 1×10^7	$P < 0.01$	$P < 0.01$	$P < 0.01$
	1×10^6 vs. 1×10^7	$P < 0.01$	$P < 0.01$	$P < 0.01$
BSA	0.04 vs. 0.4 mg	$P < 0.01$	$P < 0.01$	—
	0.04 vs. 4.0 mg	N.S.	$P < 0.01$	$P < 0.01$
	0.4 vs. 4.0 mg	$P < 0.01$	$P < 0.01$	$P < 0.01$
Hb	0.04 vs. 0.4 mg	$P < 0.01$	$P < 0.01$	N.S.
	0.04 vs. 4.0 mg	$P < 0.01$	$P < 0.01$	$P < 0.01$
	0.4 vs. 4.0 mg	$P < 0.01$	$P < 0.01$	$P < 0.01$

SE = sheep erythrocytes; BSA = bovine serum albumin; Hb = bovine hemoglobin. N.S. = not significant.

[1] Data listed in Table I were examined.

The response toward soluble proteins

The responses vary with the different proteins and the various doses (Table I).

Bovine hemoglobin consistently elicited a capsule at low doses (0.04, 0.4 mg). Tunic injury became frequent, either alone (11.1%) or with capsule (53.9%), when 4.0 mg protein was injected.

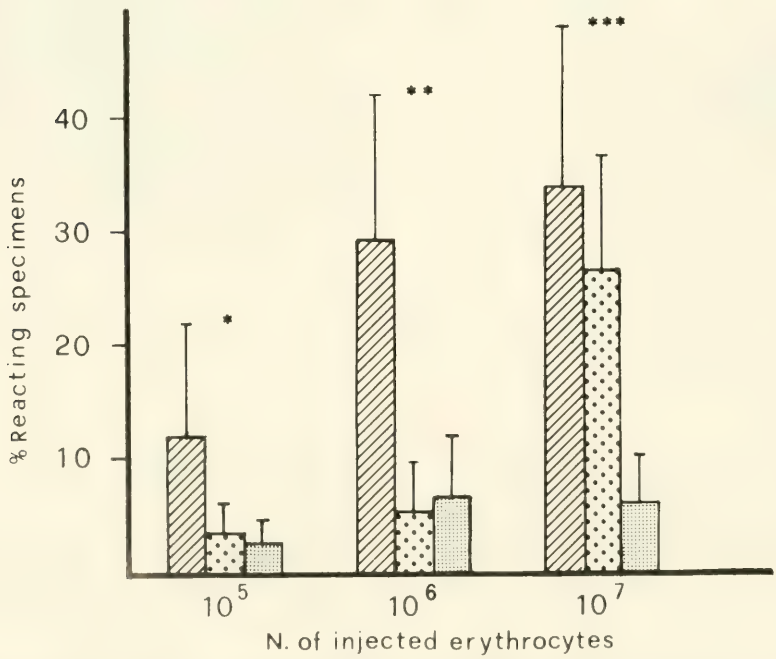

FIGURE 3. Diagram illustrating tunic reaction of *Ciona intestinalis* to increasing sheep erythrocyte doses; values are mean ± S.D., *n = 4, **n = 6, ***n = 10. ▨ = capsule, ⊡ = capsule plus injury, □ = injury.

Capsules were also produced by low doses (0.04, 0.4 mg) of bovine serum albumin but the tunic injury was the most evident response induced by this irritant. Tissue damage and a capsule were observed at 0.04 mg BSA in 19% of 100 specimens. This proportion increases to 40% at 0.4 mg. A dose of 4.0 mg BSA was consistently injurious. Injury appeared rapidly but capsules were not observed. In some specimens the injury was so severe as to produce serious tissue damage in a short time.

Hemocyanin was used in a single dose (0.4 mg) and it induced, from 4 to 7 days, tunic wounding and/or capsules in 32.1% of the 112 specimens.

Histological observations of the damaged tissue

To study the injured tunic, specimens which reacted against 1×10^7 SE or oocytes were fixed after 6 and 8 days, respectively. Additional specimens reacting in 3–4 days against 4.0 mg BSA or Hb were also prepared. Transverse and longitudinal sections, stained with Mallory's or hematoxylin-eosin stains, showed tunic lesions (Fig. 2b, c): the tunic matrix has flaked off revealing a large wound.

Granular amoebocytes (6.2–7.6 μm) are numerous and are readily identified by their eosinophil granules (0.6–0.8 μm) (Fig. 2d, e). Some of these cells can discharge their granules. This degranulation is particularly evident on the edges of the injury and can be related to the holes which occur in the tunic matrix (Fig. 2c). The degranulation mechanism can coincide with the loss of cell membrane integrity and the bulk leakage of all cell contents (Fig. 2f).

The wound produced in the tunic matrix by the injection contains particulate material. Cell infiltrate was at the wound edges. The cells appear to be granular amoebocytes which had discharged their granules. This process also occurs among the injected material masses and was particularly evident when oocytes were injected (Fig. 4). In some sections, eosinophil granulocytes are so numerous and the material in the wound so conspicuous that the deep staining masks the cells. The same features characterize the tunics injured by BSA or Hb.

To determine whether the injection procedure influenced the tunic reaction, specimens treated with PBS were studied after six days. Histological sections of the whole tunic from five specimens showed that granulocytes were rare in three specimens. In the others some of these cells were irregularly distributed near the epithelium.

Discussion

In vertebrates, inflammation is a response of living tissues to local injury. This process is characterized by local heat, swelling, redness, and pain, and leads to the accumulation of blood cells and fluid. It includes the removal of foreign materials and the disposal of damaged tissue followed by healing (Ryan and Majno, 1977). In invertebrates some features of vertebrate inflammation are not applicable. This reaction is a non-specific cellular response which includes phagocytosis, encapsulation, and wound healing (Metchnikoff, 1892). Inflammatory-like responses have been shown in annelids (Stein and Cooper, 1983), molluscs (*cfr.* Cheng, 1981), arthropods (Ratner and Vinsons, 1983), and echinoderms (*cfr.* Smith, 1981). In tunicates the encapsulation process can be provoked by natural invaders or experimentally inserted agents (*cfr.* Wright, 1981; Wright and Ermak, 1982; Wright and Cooper, 1983). The tunic reaction of *C. intestinalis* is an inflammatory-like response typical of invertebrates. Small quantities of erythrocytes injected into the tunic are cleared by phagocytosis, whereas encapsulation occurs when larger amounts are used (Wright and Cooper, 1975; Parrinello *et al.*, 1977). Increasing doses of sheep erythrocytes induce large capsules evident in the tunics of an increasing number of reacting specimens. The absence of tunic vessels probably allows for the lengthy persistence of the irritant promoting a

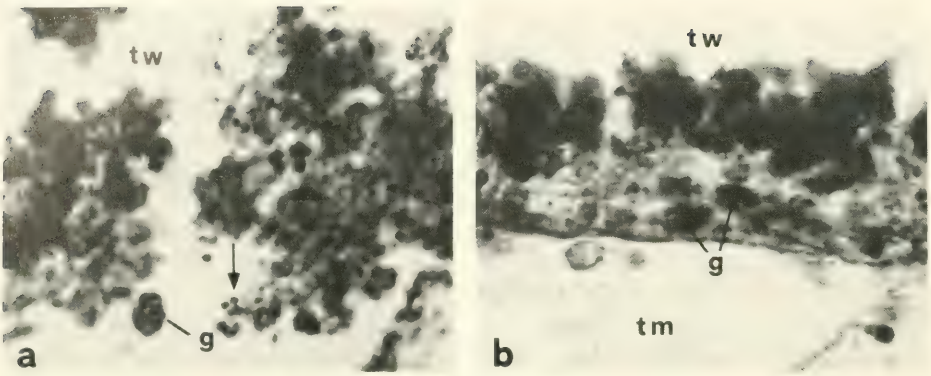

FIGURE 4. Tunic wound induced by ascidian oocyte injection. (a): Oocyte debris inside the wound, 850×; (b): granulocytes on the edge of the wound, 850×. g = granulocyte; tm = tunic matrix; tw = tunic wound; arrow indicates granules.

type of chronic inflammation. The results also suggest that encapsulation is not only elicited by particulate invaders of large size but soluble proteins can also induce capsules while large amounts of colloidal carbon or stromata fail to produce strong reactions.

The irritants that induce encapsulation can also cause tissue injury. The frequency and the seriousness of the damage, and the speed with which it occurs, are dependent on the dose and/or the nature of the irritant. Proteins seem to be more injurious agents and the highest dose of BSA induces such fast reactions (24–48 hours) that a capsule does not become visible. There is little response to stromata and colloidal carbon even in large doses.

The injury process is probably related to degranulation of the granulocytes and is not related to encapsulation. On the other hand, a capsule does not indicate a repairing process after the tissue injury. Statistically significant differences between some groups of results confirm these considerations.

Histological observation of wounds containing oocytes and SE remains suggest that the eosinophil granulocytes and the degranulation process may be involved in the removal of large degradable masses of inflammation-provoking agents. Tunic damage may be a lesser effect of a defense mechanism. It can depend on the irritant and/or dose, as shown by statistical analysis. Lysosomal mechanisms, like those proposed for neuthrophil involvement in the mammalian inflammatory reactions, could be responsible for the tunic injury (Hirschhorn, 1974; Gleisner, 1979). Agents could cause granulocyte disruption coincident with the loss of membrane integrity and possible release of lysosomal enzymes. We do not know if bacteria, either occurring naturally in the tunic (De Leo *et al.*, 1981) or resulting from contamination of the injection wound, may be involved in the tunic lysis. Moreover, cytoplasmic vacuoles of the *C. intestinalis* granulocytes can contain small deposits of vanadium associated with sulfur compounds (Rowley, 1982, 1983) which might, when released, participate in the inflammation process.

Even if the exact nature of the signal is unknown, it can be assumed that the concentration and activity of chemotactic substances could account for the strength and speed of the tunic reaction. Evidence concerning the mammalian inflammation process suggests that proteins can act directly or, after proteolysis, liberate chemotactic fragments (Houck *et al.*, 1971), while active factors may be produced by digestion of foreign material (*cfr.* Hirschhorn, 1974; Gleisner, 1979).

The contribution of naturally occurring humoral factors (bactericidins, hemagglutinins) in the non-self recognition mechanisms and their defensive role needs

further study (Wright and Ermak, 1982). The results reported in this paper suggest that hemagglutinins are not needed in the erythrocyte-induced inflammatory-like reactions, and that the inflammatory cells apparently recognize foreigness without the intervention of soluble factors.

ACKNOWLEDGMENT

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LITERATURE CITED

BECCARI, N., AND V. MAZZI. 1966. *Manuale di Tecnica Microscopica.* Soc. Ed. Libraria. 366 pp.

CHENG, T. C. 1981. Bivalves. Pp. 233–300 in *Invertebrate Blood Cells,* N. A. Ratcliffe and A. F. Rowley, eds. Academic Press, London.

DAVIS, R. F., AND S. BAKERMAN. 1972. Pp. 151–153 in *Cellular Antigens,* A. Nowotny, ed. Springer-Verlag, New York.

DE LEO, G., E. PATRICOLO, AND G. FRITTITTA. 1981. Fine structure of the tunic of *Ciona intestinalis* L. II. Tunic morphology, cell distribution and their functional importance. *Acta Zool. (Stockh.)* **62:** 259–271.

GLEISNER, J. N. 1979. Lysosomal factors in inflammation. Pp. 229–260 in *Chemical Messengers of the Inflammatory Process,* J. C. Houck, ed. Elsevier/North-Holland, Amsterdam.

HIRSCHHORN, R. 1974. Lysosomal mechanisms in the inflammatory process. Pp. 259–285 in *The Inflammatory Process,* Vol. I, B. W. Zweifach, L. Grant, and R. T. Mc Cluskey, eds. Academic Press, New York.

HOUCK, J. C., S. G. BARNES, AND C. CHANG. 1971. Pp. 39–51 in *Immunopathology of Inflammation.* Exerpta Medica Foundation, Amsterdam.

JOHNSON, P. T., AND F. A. CHAPMAN. 1970. Comparative studies on the in vitro response of bacteria to invertebrate body fluids. II. *Aplysia californica* (sea hare) and *Ciona intestinalis* (tunicate). *J. Invert. Pathol.* **16:** 259–267.

METCHNIKOFF, E. 1892. *Leçons sur la Pathologie Comparée de l'Inflammation.* Masson et Cie., Paris.

PARRINELLO, N., AND C. CANICATTÌ. 1982. Carbohydrate binding specificity and purification by biospecific affinity chromatography of *Ascidia malaca* Traust. hemagglutinins. *Dev. Comp. Immunol.* **6:** 53–64.

PARRINELLO, N., AND C. CANICATTÌ. 1983. α-Lactose binding hemagglutinins from the ascidian *Phallusia mamillata* (Cuv.). *Biol. Bull.* **164:** 124–135.

PARRINELLO, N., AND E. PATRICOLO. 1975. Erythrocyte agglutinins in the blood of certain ascidians. *Experientia* **31:** 1092–1093.

PARRINELLO, N., E. PATRICOLO, AND C. CANICATTÌ. 1977. Tunicate immunobiology. I. Tunic reaction of *Ciona intestinalis* L. to erythrocyte injection. *Boll. Zool.* **44:** 373–381.

PARRINELLO, N., AND E. PATRICOLO. 1984. Inflammatory-like reaction in the tunic of *Ciona intestinalis* (Tunicata). II. Capsule components. *Biol. Bull.* **167:** 238–250.

RATNER, S., AND S. B. VINSON. 1983. Phagocytosis and encapsulation: Cellular immune responses in Arthropoda. *Am. Zool.* **23:** 185–194.

REDDY, A. L., B. BRYAN, AND W. H. HILDEMANN. 1975. Integumentary allografts versus autografts reactions in *Ciona intestinalis.* A protochordate species of solitary Tunicate. *Immunogenetics* **1:** 584–590.

ROWLEY, A. F. 1982. The blood cells of *Ciona intestinalis:* an electron probe X-ray microanalytical study. *J. Mar. Biol. Assoc. U.K.* **62:** 607–620.

ROWLEY, A. F. 1983. Preliminary investigations on the possible anti-microbial properties of tunicate blood cell vanadium. *J. Exp. Zool.* **227:** 319–322.

RYAN, G. B., AND G. MAJNO. 1977. Acute inflammation. *Am. J. Pathol.* **86:** 185–278.

SMITH, V. J. 1981. The echinoderms. Pp. 513–562 in *Invertebrate Blood Cells,* N. A. Ratcliffe and A. F. Rowley, eds. Academic Press, London.

STEIN, E. A., AND E. L. COOPER. 1983. Inflammatory responses in annelids. *Am. Zool.* **23:** 145–156.

WRIGHT, R. K. 1981. Urochordates. Pp. 565–626 in *Invertebrate Blood Cells,* N. A. Ratcliffe and A. F. Rowley, eds. Academic Press, London.

WRIGHT, R. K., AND E. L. COOPER. 1975. Immunological maturation in the tunicate *Ciona intestinalis. Am. Zool.* **15:** 21–27.

WRIGHT, R. K., AND E. L. COOPER. 1983. Inflammatory reactions of the Protochordata. *Am. Zool.* **23:** 205–211.

WRIGHT, R. K., AND T. H. ERMAK. 1982. Cellular defense systems of the Protochordata. Pp. 283–320 in *The Reticuloendothelial System, a Comprehensive Treatise. Phylogeny and Ontogeny,* Vol. 3, N. Cohen and M. M. Sigel, eds. Plenum Press, New York.

Reference: *Biol Bull.* **167:** 238–250. (August, 1984)

INFLAMMATORY-LIKE REACTION IN THE TUNIC OF *CIONA INTESTINALIS* (TUNICATA). II. CAPSULE COMPONENTS

NICOLÒ PARRINELLO AND ELEONORA PATRICOLO

Institute of Zoology, Palermo University, Via Archirafi, 18 90123 Palermo, Italy

ABSTRACT

An inflammatory-like process is initiated in the tunic of *Ciona intestinalis* by particulate agents and soluble proteins. It includes the induction of a capsule in the form of a whitish disc in the tunic and which isolates injected materials. Histologically, the capsule structure shows a large number of cells collected around the injection wound. Vesicular cells release an unidentified amorphous substance which mixes with mucopolysaccharide and protein contents of typical "granule-packed cells" to form the capsule matrix. The vacuolated epithelial cells, lining the inner zone of the tunic, can release an unidentified substance which participates in matrix production. Lymphocyte-like cells can be present and transitional cells may differentiate into the vesicular cells. Large refringent granule cells, and compartment cells which can contain refringent material, were found inside the wound and are thought to release the glycoprotein substances. The degranulation of eosinophil granulocytes occurs in the same period. The capsule induced by soluble proteins lacks "granule-packed cells" while fine granular material, mucopolysaccharide in nature, is layered on the epithelium of the capsule. Observations of early stages of capsule formation show granular and hyaline amoebocytes, probably phagocytes, which quickly surround the foreign material (*e.g.*, erythrocytes) followed by transitional cells and, finally, vesicular cells, which can form an incipient capsule within 24 hours. Encapsulation in *Ciona intestinalis* is a cellular response which includes the mechanisms for tunic production in order to isolate the inflamed tissue while the foreign material is disrupted and the injection wound repaired.

INTRODUCTION

In tunicates, encapsulation is a chronic inflammatory-like response which appears, in the body wall, to isolate natural invaders (Bresciana and Lützen, 1960; Monniot, 1963; Dudley, 1968), experimentally inserted objects (Smith, 1970; Anderson, 1971), or bacteria (Thomas, 1931). Vacuolated blood cells, predominantly morula cells, are responsible for these reactions (Wright, 1981; Wright and Ermak, 1982).

The ascidian, *Ciona intestinalis,* reacts by cellular responses toward foreign materials inserted into the tunic (Wright and Cooper, 1975; Parrinello *et al.*, 1976, 1977). In previous papers we have shown that a capsule can envelop the particulate or soluble injected materials by cell infiltration and glycoprotein secretions (Parrinello *et al.*, 1976, 1977). In some specimens the response can induce local tissue damage which seems to be independent of encapsulation. The tunic injury depends on the dose and nature of the irritant, involves eosinophil granulocytes, and shows lysosomal content release by various degranulation mechanisms (Parrinello and De Leo, in prep.).

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Abbreviations: SE = sheep erythrocytes; PBS = phosphate-buffered saline (0.01 *M* pH 7.4 phosphate buffer containing 0.15 *M* NaCl); BSA = bovine serum albumin; Hc = *Octopus vulgaris* hemocyanin; Hb = bovine hemoglobin; PAS = periodic acid Schiff.

The *C. intestinalis* capsule is a whitish disc around the injected material and is visible through the transparent tunic. Each treated specimen can react in various degrees to the foreign matter and the intensity of the response is dependent upon both the nature and the dose of the eliciting agent (Parrinello *et al.*, 1984). Preliminary histological observations of capsules obtained after erythrocyte injection showed numerous univacuolated cells and packed granular materials in the inflamed area. In the present paper the histopathology of the capsules produced after injection of particulate or soluble agents is described, and data are also reported on the stages in differentiation and role of some capsular components.

Materials and Methods

Adult *Ciona intestinalis* L. specimens (~10–12 cm in length) were collected from Palermo harbor. Animals with tunics free of marine matter were selected and maintained at 15–18°C in glass tanks containing adequately aerated sea water.

Injection of animals

The following particulate material suspensions were prepared in phosphate-buffered saline at pH 7.4 (PBS): 5×10^7/ml sheep erythrocytes (SE), 2 and 20 mg/ml colloidal carbon (G. Wagner, Hannover, Lot C 11/1431 A), and 10% (v/v) sheep red cell membranes prepared according Davis and Bakerman (1972). The response to a more complex cellular system was investigated by injecting *C. intestinalis* oocytes collected from several specimens, washed, and suspended in PBS ($1.0–1.2 \times 10^3$ oocytes/ml). Bovine serum albumin (BSA) (Sigma), bovine hemoglobin (Hb) (Sigma), and *Octopus vulgaris* hemocyanin (Hc) (kindly supplied by Dr. G. Nardi, Zoological Station, Naples) were used at the concentration (2 mg/ml) which is known (Parrinello *et al.*, 1984) to produce a visible capsule in the *C. intestinalis* tunic. A 0.2 ml volume was injected, using a syringe and 27-gauge needle, into the tunic under the cuticle in the region of the gut loop. Control specimens were injected with 0.2 ml PBS.

Light microscopy

Portions of the tunic from the injected area, whether showing a capsule or not, and the corresponding region of the untreated or PBS-injected specimens, were fixed in 70% ethanol and embedded in paraffin wax or paraplast. Histochemical reactions were performed on 7 μm sections using techniques reported by Beccari and Mazzi (1966), Luna (1968), and Ganter and Jollès (1970). Mallory's trichrome and hematoxylin-eosin stains were employed in general cytological studies to identify tunic zones and cells, Millon's reaction was used to detect proteins, the periodic acid-Schiff (PAS) test for polysaccharides, and 1% alcian blue stain (pH 1.0 and 2.5) for mucopolysaccharides. Pickworth's method with benzidine for hemoglobin was used.

Some of the cell types in the inflamed tunic correspond to cells described by other authors using light and electron microscopy techniques on normal tissues of *C. intestinalis*. Their definitions are: (1) lymphocyte, blood cell (3–5 μm) with a high nuclear:cytoplasmic ratio (Millar, 1953; Rowley, 1981); (2) vesicular cell, blood and tunic cell (5–7 μm) with a single large vacuole occupying most of the cell and forcing the discoid nucleus to one side, the shape can be signet-ring-like (Millar, 1953); (3) morula cell, round or elliptical shaped cell (6–8 μm) found in both the blood and the tunic, containing several globules around the cell periphery, the nucleus often cannot be distinguished (De Leo *et al.*, 1981; Rowley, 1981); (4) large refringent granule cell, tunic cell (4–6 μm) with a single large refringent granule which occupies almost all the volume of the cell, the nucleus is displaced in a cytoplasmic polar cup

(*cfr.* De Leo *et al.*, 1981); (5) granular amoebocyte, blood cell (13.4 × 7.3 μm, mean values) showing cytoplasm with a variable number of granules and occasional vacuoles; protoplasmic extensions are evident (Rowley, 1981); (6) hyaline amoebocyte, blood cell (6.7 × 15.9 μm, mean values) showing cytoplasm with a number of large vacuoles, is highly amoeboid, and often has lobose pseudopodia (Millar, 1953; Rowley, 1981), both types of amoebocytes were also found in the tunic (Millar, 1953; De Leo *et al.*, 1981); (7) mantle epithelium cells, they are polygonal cells which form a flattened single layered epidermis immediately under the tunic, their cytoplasm is weakly stained and their nuclei has scattered chromatin blocks, a vacuole can occupy part of the cell (Millar, 1953).

Results

The tunic of untreated and PBS-injected specimens

To compare the normal with treated tunics, serial transverse sections from pharingeal and gut-loop regions of five untreated specimens were first examined.

The tunic consists of fibers (polysaccharides and proteins) embedded in an amorphous ground substance (acid mucopolysaccharides and low concentrated proteins) and cells (De Leo *et al.*, 1977; Patricolo and De Leo, 1979; De Leo *et al.*, 1981). The tissue is organized in a gelatinous "inner zone" and a dense "outer zone" showing an external glycoprotein cuticle and an internal single-layered epithelium.

The cell types (see Methods) and their frequencies (numbers based on counts of each optical field at 315×) can vary.

Large refringent granule cells and morula cells, usually observed in the outer layer, can occasionally be found in the inner zone; they were rare in three specimens.

Granulocytes, round, elliptical, or amoeboid (granular amoebocyte) in shape, can be scattered variously throughout the tunic: 2–3 cells per optical field were found in three specimens, while 10–15 cells were near the epithelium in two.

Vesicular cell, signet-ring-like, show a single large vacuole full of unstained material which reduces the cytoplasm to a peripheric strip. They were rare in two specimens and absent in one; 5–9 cells per optical field were found in two specimens.

Lymphocytes were only found (2–4 cells per optical field) in a few tunic areas near the epithelium of two specimens.

The cells of the epithelium show a vacuole containing unstained material.

To investigate the mechanical effect of the liquid injection, histological sections of tunic fragments were examined at different time intervals after 0.2 ml PBS injection. A tunic reaction was never observed. Initially the saline injection produced a wound in the tunic matrix which then healed; in fact it was not observed in two specimens fixed after 4–6 hours. No unusual distribution or frequency of cell types were found in this area even when two different tunic fragments were examined 12 hours and 5 days after the injection, respectively.

Capsule structure

Following injection of particulate material or proteins into the tunic of *C. intestinalis* a capsule (1.5–3.0 cm wide) can appear within 2–8 days (Fig. 1a). The appearance time is variable depending on dose, nature of the eliciting agent, and, probably, on the animal lot used in each experiment (Parrinello *et al.*, 1984).

Capsule induced by particulate materials. Figure 1 (b, c) shows transverse sections of the tunic seven days after injection of *C. intestinalis* oocytes. The injected material is contained in a tunic wound, while cells infiltrate the inflamed area apparently

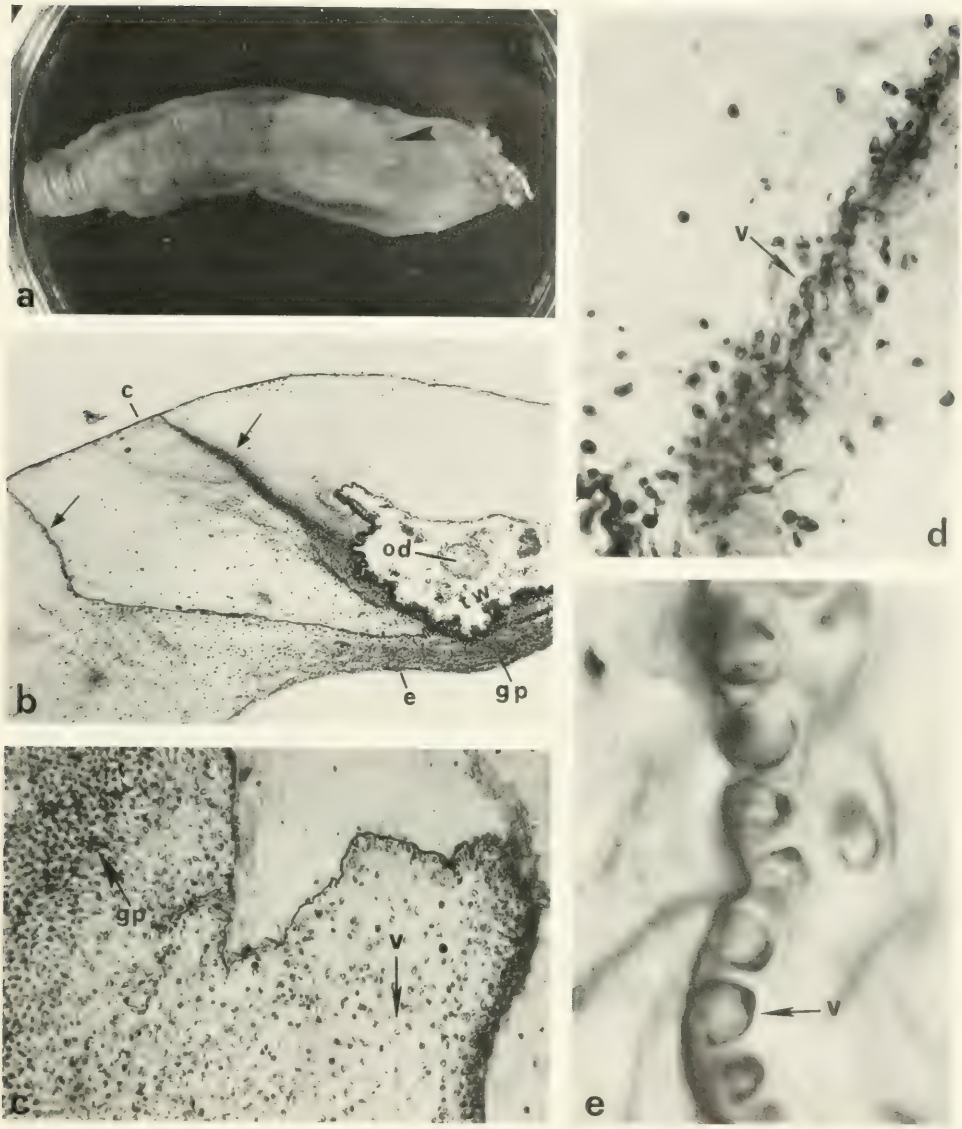

FIGURE 1. Encapsulation of ascidian oocytes injected into *Ciona intestinalis* tunic. (a) Capsule seven days after injection (arrowhead). (b–e) Transverse sections of the tunic containing capsule (Mallory's stain); (b) arrows indicate the lined up cells which envelop the foreign material, 40×; (c) closer view of the capsule 470×; (d) amoeboid vesicular cells (v) forming the capsular outline, 630×; (e) vesicular cells which release vacuolar content to form an encapsulating strip, 1420×. c, cuticle; e, epithelium; gp, granule-packed cells; od, oocyte debris; tw, tunic wound.

originating from the tissue under the epithelium to enclose the injured tunic up to the cuticle (Fig. 2).

Histological observations of tunic fragments from five specimens injected with 1×10^7 SE and two specimens injected with oocytes, showed that cells accumulated around the wound in large bands in the tunic, from the epithelium to the foreign

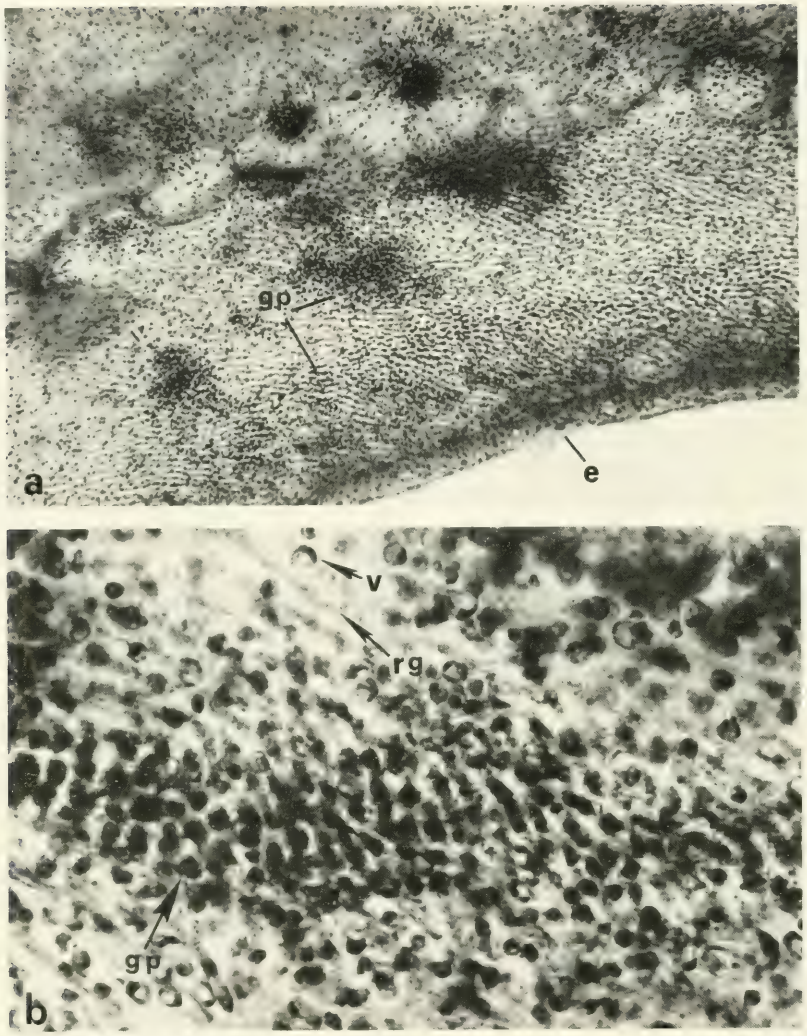

FIGURE 2. Capsule transverse sections seven days after sheep erythrocyte injection into *Ciona intestinalis* tunic (Mallory's stain). (a) Tunic inner zone lined by epithelium (e), 95×. (b) Closer view showing granule-packed cells (gp), released granular material (rg) and vesicular cells (v), 570×.

material. They are called "granule-packed cells" because their cytoplasm is completely filled with strongly aniline blue (Mallory's trichrome) stained fine granules which mask the nucleus (if present); their diameters range from 2.1 to 5.6 μm (Fig. 3a–c). The granules are positive to Millon, PAS, and alcian blue reactions. In some cells they are less packed and more easily distinguished, in other cells the granular material apparently dissolves while cells are elongated and stretch out releasing their contents into the tunic matrix.

In three specimens injected with erythrocytes, granule-packed cells were found in close contact with the epithelium and up to the inner border of the wound. In longitudinal sections they were arranged in 2–3 concentric layers around the wound.

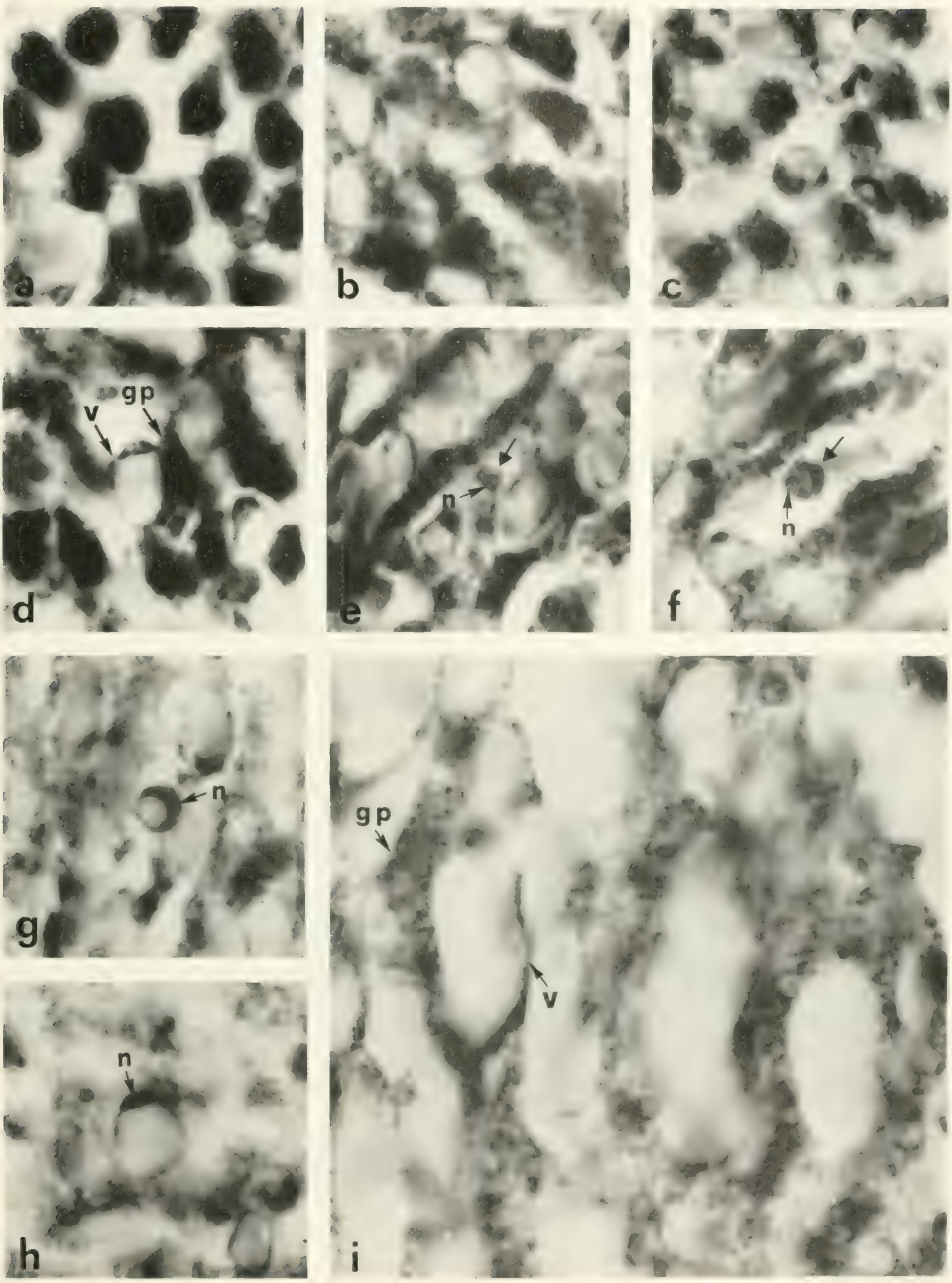

FIGURE 3. Capsular components seven days after erythrocyte injection into *Ciona intestinalis* tunic, 1420× (Mallory's stain). (a–c) Various features of granule-packed cells. (d) Vesicular cells (v) and granule-packed cells (gp) in close contact. (e) Lymphocyte (arrow) among vesicular cells and elongated granule-packed cells. (f) Transitional cell (arrow). (g) Immature vesicular cell (arrow). (h) Large vesicular cells releasing amorphous material. (i) End stages of granule-packed cells and vesicular cells. n, nucleus.

Vesicular cells filled the reacting tunic particularly at the inner edge of the wound. They range in size from 3.5 to 7.1 μm and vary in appearance according to their stage of development. These cells can assume an amoeboid shape and fall into line to form the capsular outline and encapsulating strip, some release their amorphous contents by dissolving a portion of their membrane (Fig. 1d, e). The vacuolar substance was negative with Mallory's stain, PAS, Millon, and alcian blue reactions.

Granule-packed cells and vesicular cells can be in close contact; their membranes apparently dissolve and their contents mix (Fig. 3d, i). Both cell types appear to contribute to the production of capsular substance. In some zones, the granular material has a streaked appearance due to ghosts of the large vesicular cells.

Small lymphocytes (2.1–3.4 μm) with an aniline blue slightly cytoplasm (Fig. 3e), are present in the reacting tunic.

Spherical cells, 3.2–4.5 μm in diameter, with an eccentric or central nucleus and aniline blue staining cytoplasm are considered transitional cells. They are frequent among the granule-packed cells and are numerous near the epithelium as far as the inner edge of the wound. Figure 3 (e–g) shows the presumptive stages in the vesicular cell differentiation.

The cells of the mantle epithelium (9.2–11.3 μm, in surface view), show vacuoles 3.5–7.4 μm wide containing an amorphous substance which is negative to Mallory's trichrome, PAS, Millon, and alcian blue histochemical reactions. Large vacuoles can release their contents into the tunic matrix by dissolving a portion of their membranes. Multi-layered epithelium was observed in some capsule transverse sections (Fig. 4).

Granulocytes (6.2–7.6 μm) are distributed along the edges and inside the wound; they contain eosinophil granules 0.6–0.8 μm in diameter and can degranulate, releasing the latter by cell membrane dissolution. In wounds produced by erythrocyte injection, the intensely stained material distributed along the edges makes it difficult to identify the granulocytes.

Large round cells (7.5–8.5 μm), probably phagocytes, containing vacuoles full of aniline blue-staining material, are frequently found at the edges of the wound. These vacuoles occupy the cytoplasm and contain substances which are PAS and Millon positive but alcian blue negative. The nucleus is indistinguishable (Fig. 5a).

Large refringent granule cells (4.5–6.4 μm) are numerous in the wound. A single large orange-stained granule occupies almost all of the cell (Fig. 5b). Cells with a smaller refringent granule show more evident semilunar cytoplasm. Other cells release their granule by dissolving a portion of the cell membrane. These last two cell types are not frequent in the tunic wound containing oocytes.

Compartment cells (6.3–7.9 μm) are characterized by 2–4 vacuoles separated from each other by cytoplasmic partitions (Fig. 5c); they are similar to those described in the blood of other ascidians (*cfr.* Wright, 1981). The vacuolar contents can be slightly stained by aniline blue or appear as yellow stained refringent inclusions; in some cells a prominent refringent inclusion can occupy a large part of the cytoplasm (Fig. 5d). The nucleus is not visible and the cell outlines are often obscure (Fig. 5e, f). Free yellow refringent granules (2.1–2.8 μm) can be found (Fig. 5g). Large numbers of compartment cells and free granules populate the wound.

The specimens injected with stromata (1% or 10%) or colloidal carbon (2 or 20 mg/ml) never showed an evident capsule. However histological investigations revealed some capsular components in the injected area. Within 6–7 days vesicular cells in various differentiation stages were distributed in the tunic matrix between the epithelium and the inner edge of the wound. Round phagocytes full of deeply aniline blue-stained material or carbon masses, and large refringent granule cells are scattered

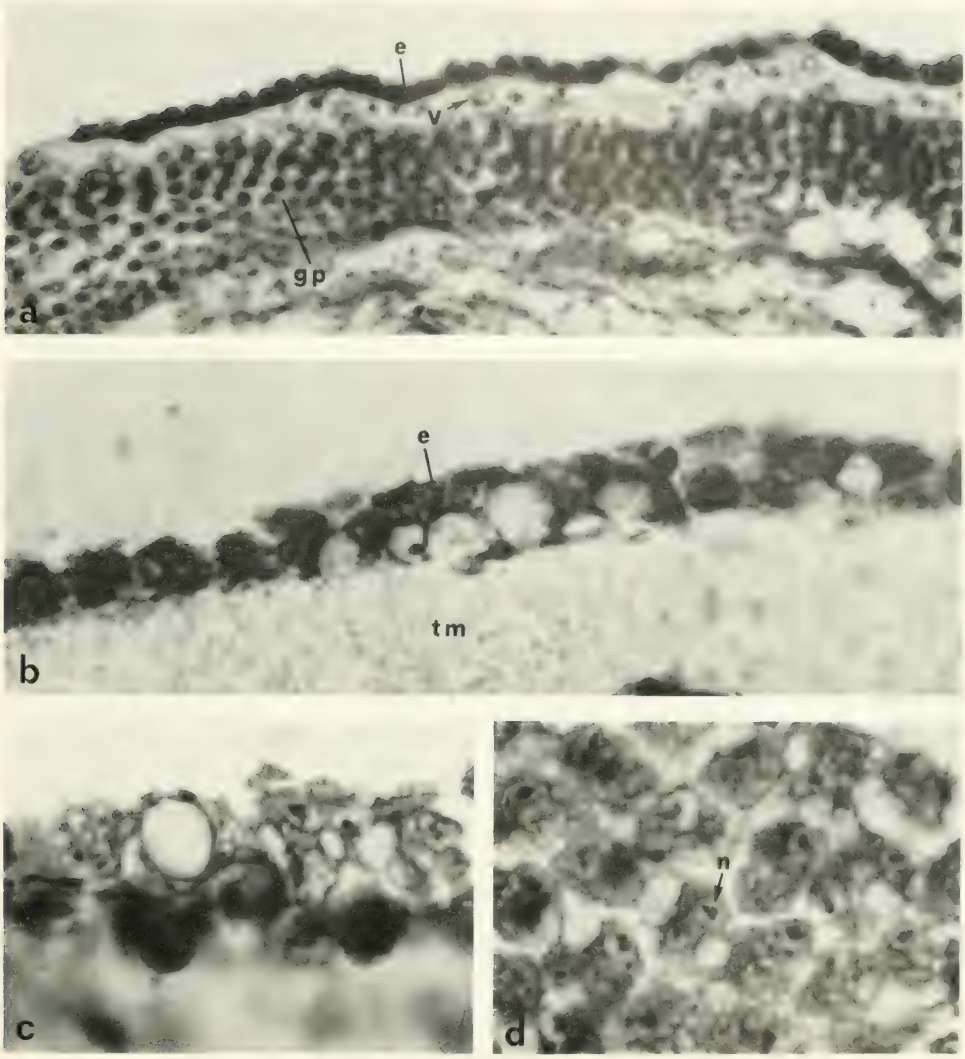

FIGURE 4. Epithelium and inner zone of *Ciona intestinalis* tunic after sheep erythrocyte injection (Mallory's stain). (a) Transverse sections showing single layered epithelium (e) and granule-packed cells (gp), 570×. (b, c) Active epithelium releasing vacuolar content, 1420×. (d) Surface view of the epithelial cells, 1420×. v, vesicular cell; n, nucleus; tm, tunic matrix.

among the foreign materials. When the highest doses were used these cells were more numerous.

Capsule induced by soluble proteins. Histological transverse sections of capsules obtained 6 days after 0.4 mg protein (BSA, Hc, Hb) injection, showed that the tunic around the wound is populated with vesicular cells varying in differentiation stages. Inside the wound, aniline blue-stained large round cells and large refringent granule cells are frequent; granulocytes are settled on the inner edge, where they can degranulate.

Fine granular materials (PAS, Millon, and alcian blue positive) were layered on

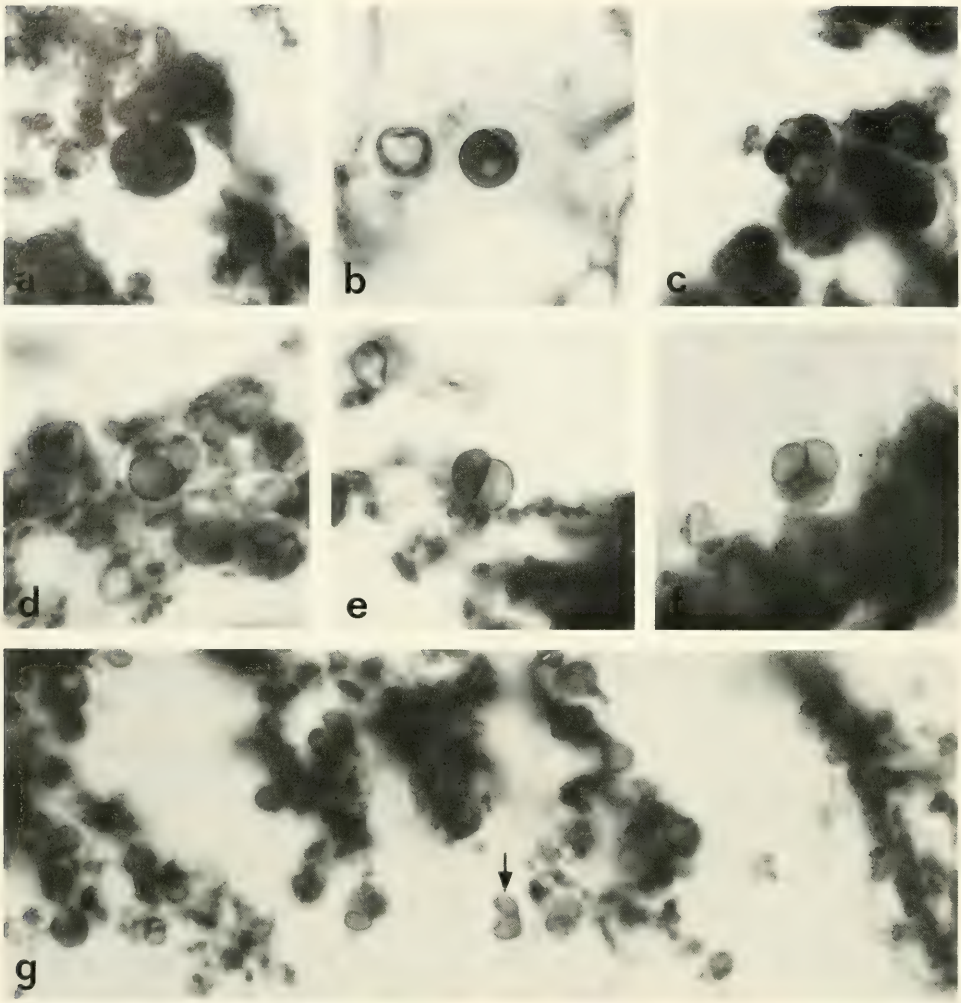

FIGURE 5. Cells inside the tunic wound seven days after erythrocyte injection; erythrocyte debris is indistinguishable, 1420× (Mallory's stain). (a) Large round phagocyte. (b) Large refringent granule cell. (c) Compartment cell. (d–f) Compartment cell with refringent inclusions. (g) Free refringent globules (arrow) inside the wound.

the epithelium forming a band which gradually decreased in thickness toward the capsule margin.

Early stages in the tunic reaction

Histological study of the tunic showed that rapid cellular responses follow erythrocyte injection. After 2–4 hours vesicular cells in various differentiation stages predominate near the epithelium. Aniline blue-stained large round phagocytes were scattered among the erythrocytes; the latter formed large masses at the inner edges of the wound.

In tunics fixed after 12–16 hours (Fig. 6a, c–e) many hyaline and granular amoebocytes (7.1–11.2 × 2.3–3.5 μm) formed a band along the inner wound edge and

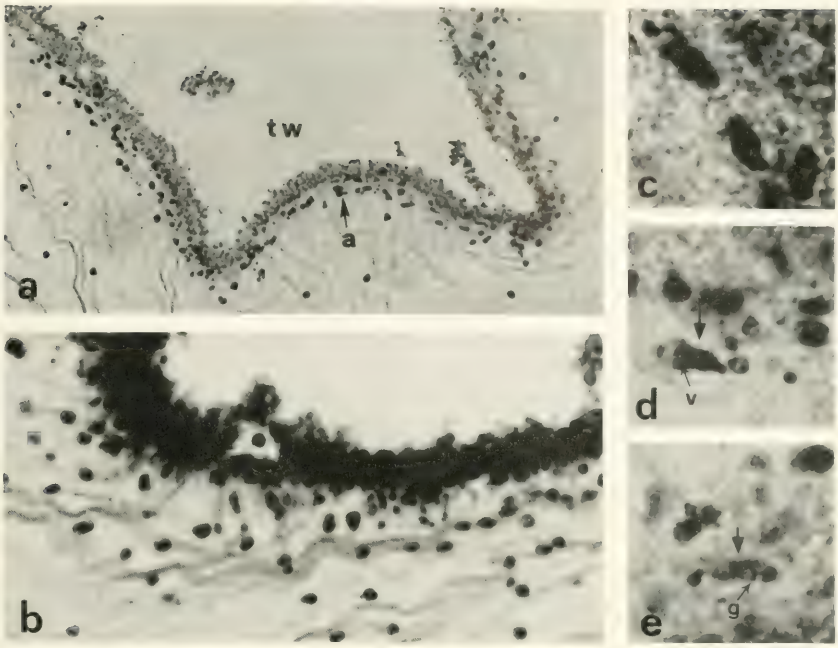

FIGURE 6. Early response in the tunic reaction of *Ciona intestinalis* after erythrocyte injection. (a, b) Transverse sections showing tunic wound (tw) and amoebocyte (a) infiltration. (a) Twelve hours after injection, 160×. (b) Twenty-four hours after injection, 390×. (c) Benzidine-positive amoebocytes, 980×. (d) Hyaline amoebocyte (arrow), 980×. (e) Granular amoebocyte (arrow), 980×. g, granule; v, vacuole. a, b, d, e: Mallory stained.

showed lobose pseudopodia. Some vesicular cells lined up forming an encapsulating strip while others filled the tunic matrix and may be amoeboid in shape. Large refringent granule cells are numerous inside the wound and are also present in the inner tunic layer below the wound. Nodules of transitional cells are observed near the epithelium.

In tunics fixed after 24 hours, the above cells are more numerous. On the wound inner edge, the cells are full of aniline blue-stained material (Fig. 6b) while the erythrocyte masses are not easily distinguished.

After 48 hours many compartment cells, with vacuoles containing yellow refringent material alone were found inside the wound. Granule-packed cells were also present in the tunic while large vacuoles characterized the epithelial cells.

At 12 and 24 hours the large round cells inside the wound and the amoebocytes were positive to the benzidine histochemical reaction for hemoglobin (Fig. 6c). Tests carried out at 48 hours and 6 days were negative.

The tunics from specimens which 6–7 days after SE injection did not show an apparent capsule, presented in the injection site the same components found in the early stages (12–48 hours) of the capsule development. Low general cell frequency and few granule-packed cells characterize these tunic areas.

The capsule increases in thickness and 12–20 days after 5×10^7 SE injection, forms a protuberance of the tunic and a decreased number of cells. The wound was reduced, probably by coalescence of the edges which appear partially free of cells and material. Encapsulating strips, when present, contained few vesicular cells.

A quick response followed stromata or colloidal carbon injection. Vesicular cells

formed thin encapsulating strips in the tunic fixed after 24–48 hours. Small carbon masses were in the round phagocytes inside the wound. Hyaline and granular amoebocytes with carbon inclusions, formed a band along the inner wound edge. Granule-packed cells or free granular material were never observed in the tunic.

Discussion

Ciona intestinalis is capable of recognizing non-self materials and reacts by cellular responses. Foreign agents inserted into the tunic, including tunic tissue allografts, are removed from the body (Reddy *et al.*, 1975; Wright and Cooper, 1975; Parrinello *et al.*, 1977). These reactions are considered to form part of the internal defense system. They show characteristics of inflammatory-like processes including phagocytosis, encapsulation, and tissue damage.

Encapsulation is a response elicited by both corpuscular material and soluble proteins (Parrinello *et al.*, 1984). The nature and dose of the foreign agent determine the strength of the response and influence the structure of the capsule which is visible in the most reactive specimens. Early stages of encapsulation also occur in the tunic of apparently non-reactive animals; the absence of granule-packed cells or granular material lining the active epithelium could account for the non-appearance of the capsule.

The removal of the foreign material and tunic debris may be rapidly effected by phagocytes. These cells correspond reasonably well to the blood hyaline and granular amoebocytes described by Rowley (1981). They may begin the tunic reaction by performing non-self recognition. The nature of the cell contents in the responses to various agents suggests that the large round cells inside the wound could also be phagocytes. The removal of large masses may be facilitated by a lysosomal mechanism dependent on the eosinophil granulocyte degranulation. Large numbers of these cells were found in the wound containing oocytes, where the resultant degraded material may induce further inflammation (*cfr.* Hirschhorn, 1974; Gleisner, 1979). Such activity may also lead to tunic injury (Parrinello *et al.*, 1984).

Lymphocytes can infiltrate the inflamed tunic, however their quantity is not important to capsule composition. The transitional cells are numerous. They are a first stage in which stem cells differentiate to produce capsule cells. From morphological evidence the presumptive stages in vesicular cell differentiation are as described in the blood by Millar (1953). Lymphocytes and transitional cells as well as granular and hyaline amoebocytes may originate from the blood or from the nodules of hemopoietic tissue situated in the pharingeal wall and around the gut loop (Ermak, 1976).

An unidentified amorphous substance contributes to the manufacture of the capsule matrix. It is contained in large vacuoles of mantle epithelium cells and vesicular cells. It is released by vacuole and cell membrane dissolution. Vesicular cells rapidly fill the inflamed area and line up in a continous layer, isolating the injured tissue. In this respect encapsulation may involve the mechanisms that construct or regenerate the tunic. Enlargement and vacuolization of epithelial cells and cell types ("cell with a small acidophil vacuole" and "phagocyte univacuolated"), probably corresponding to differentiation stages of vesicular cells, have been described by Pérès (1948) in the tunic regeneration of *C. intestinalis.* Moreover, in the ascidian tunic, epithelial cells take up the glucose used in ground substance production. In *C. intestinalis* this monosaccharide is used in the manufacture of acid mucopolysaccharides, and may be incorporated into the tunic fibers (Robinson *et al.*, 1983). Evidence for the cellulose nature of this capsular material is not available, but histochemical evidence suggests

that it collaborates with mucopolysaccharides in tunic matrix formation. This was particularly evident when the fate of the vesicular cells and the granule-packed cells was observed. The packed fine granules consisting of mucopolysaccharides gradually dissolve and apparently mix with the vesicular cell products. The origin of the granule packed cells and fine granular material is unknown. They might have an epithelial origin because they frequently appear in close contact with the epithelium.

Large refringent granule cells have been described in the regeneration of the tunic as occasionally occurring across the epithelium (Pérès, 1948) and usually present just below the cuticle. They may be involved in the production of this glycoprotein layer (De Leo *et al.*, 1981) and may produce the glycoproteins inside the wound and contribute to the healing by cuticular material formation.

Compartment cells could be stages in morula cell development as proposed for the corresponding blood cells in other ascidian species (Endean, 1960; Kalk, 1963; Smith, 1970). The absence of mature morula cells in the inflamed area could depend on the activity of lysosomal enzymes which interrupt cell development and induce granule release. It is known that morula cells congregate and break down at the edge of wounds produced by extirpating fragments of *Halocynthia aurantium* tunic (Smith, 1970). Moreover, they form the capsule which surrounds glass fragments inserted into the tunic of *Molgula manhattensis* (Anderson, 1971).

Differences in the cell composition of the capsules apparently depend on the nature and size of the irritant. Apart from the granule-packed cells and round phagocytes, the other cells are also found in the normal or PBS-injected tunic. They are variably distributed and definitely less frequent. As yet the origin of the capsule cells is unknown.

The contribution of naturally occurring humoral factors in the ascidian non-self recognition mechanisms is unclear. However, hemagglutinins are probably not involved in the erythrocyte tunic encapsulation of *C. intestinalis* (Parrinello *et al.*, 1984).

In summary, the tunic reaction of *C. intestinalis* is a response consisting of several processes: (1) non-self recognition by phagocytes which can intervene in early stages of the reaction; (2) degranulation of eosinophil granulocytes which can disrupt large foreign material, probably by lysosomal mechanisms which in some cases may cause tissue damage (Parrinello *et al.*, 1984); (3) tunic matrix substance production with formation of a thick capsule to isolate the inflamed area; and (4) cuticular glycoprotein production to heal the tunic wound.

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LITERATURE CITED

ANDERSON, R. S. 1971. Cellular responses to foreign bodies in the tunicate *Molgula manhattensis* (De Cai). *Biol. Bull.* **141:** 91–98.

BECCARI, N., AND V. MAZZI. 1966. *Manuale di Tecnica Microscopica.* Soc. Ed. Libraria. 366 pp.

BRESCIANA, J., AND J. LÜTZEN. 1960. *Gonophysema gullmarensis* (Copepoda Parasitica). An anatomical and biological study of an endoparasite living in the Ascidian *Ascidiella aspersa.* I. Anatomy. *Cac. Biol. Mar.* **1:** 157–184.

DAVIS, R. F., AND S. BAKERMAN. 1972. Pp. 151–153 in *Cellular Antigens,* A. Nowotny ed., Springer-Verlag, New York.

DE LEO, G., E. PATRICOLO, AND G. D'ANCONA LUNETTA. 1977. Studies on the fibrous components of the test of *Ciona intestinalis* Linnaeus. I. Cellulose-like polysaccharide. *Acta Zool. (Stockh.)* **58:** 135–141.

DE LEO, G., E. PATRICOLO, AND G. FRITTITTA. 1981. Fine structure of the tunic of *Ciona intestinalis* L.

II. Tunic morphology, cell distribution and their functional importance. *Acta Zool. (Stockh.)* **62:** 259–271.

DUDLEY, T. L. 1968. A light and electron microscopic study of tissue interactions between a parasitic copepod, *Scoledocus huntsmani* (Henderson), and its host ascidian *Styela gibbsi* (Stimpson). *J. Morphol.* **124:** 263–281.

ENDEAN, R. 1960. The blood cells of the ascidian *Phallusia mammillata. Q. J. Microsc. Sci.* **101:** 177–197.

ERMAK, T. H. 1976. The hematogeneic tissues of tunicates. Pp. 45–56 in *Phylogeny of Thymus and Bone Marrow-Bursa Cells,* R. K. Wright and L. C. Cooper, eds. Elsevier/North-Holland, Amsterdam.

GANTER, P., AND G. JOLLÈS. 1970. *Histochemie Normale et Pathologique,* Vol. 2. Gauthier-Villars, Paris.

GLEISNER, J. M. 1979. Lysosomal factors in inflammation. Pp. 229–260 in *Chemical Messengers of the Inflammatory Process,* J. C. Houck, ed. Elsevier/North-Holland, Amsterdam.

HIRSCHHORN, R. 1974. Lysosomal mechanisms in the inflammatory process. Pp. 259–285 in *The Inflammatory Process,* Vol. I, B. W. Zweifach, L. Grant, and R. T. Mc Cluskey, eds. Academic Press, New York.

KALK, M. 1963. Intracellular sites of activity in the histogenesis of tunicate vanadocytes. *Q. J. Microsc. Sci.* **104:** 483–493.

LUNA, L. G. 1968. *Manual of Histologic Staining Methods,* of the Armed Forces Institute of Pathology. McGraw-Hill, New York. 258 pp.

MILLAR, R. H. 1953. Ciona. *L.M.B.C. Memoirs on Typical British Marine Plants and Animals.* **35:** 1–123.

MONNIOT, C. 1963. *Kystodelphys drachi* n.g.n. sp., copepode enkyste dans une branchie d'Ascidie. *Vie Milieu* **14:** 263–273.

PARRINELLO, N., AND E. PATRICOLO. 1975. Erythrocyte agglutinins in the blood of certain ascidians. *Experientia* **31:** 1092–1093.

PARRINELLO, N., G. DE LEO, AND E. PATRICOLO. 1976. Evolution of the immune response. Tunic reaction of *Ciona intestinalis* L. to erythrocyte injection. Some ultrastructural aspects. *Boll. Zool.* **43:** 390.

PARRINELLO, N., E. PATRICOLO, AND C. CANICATTÌ. 1977. Tunicate immunobiology. I. Tunic reaction of *Ciona intestinalis* L. to erythrocyte injection. *Boll. Zool.* **44:** 373–381.

PARRINELLO, N., E. PATRICOLO, AND C. CANICATTÌ. 1984. Inflammatory-like reaction in the tunic of *Ciona intestinalis* (Tunicata). I. Encapsulation and tissue injury. *Biol. Bull.* **167:** 229–237.

PATRICOLO, E., AND G. DE LEO. 1979. Studies on the fibrous components of the test of *Ciona intestinalis* Linnaeus. II. Collagen-elastin-like protein. *Acta Zool. (Stockh.)* **60:** 259–269.

PÉRÈS, J. M. 1948. Recherches sur la genèse et la régénération de la tunique chez *Ciona intestinalis* L. *Bull. Inst. Oceanogr. (Monaco)* **936:** 1–12.

REDDY, A. L., B. BRYAN, AND W. H. HILDEMANN. 1975. Integumentary allografts versus autografts reactions in *Ciona intestinalis:* A protochordate species of solitary Tunicate. *Immunogenetics* **1:** 584–590.

ROBINSON, W. E., K. KUSTIN, AND G. C. MCLEOD. 1983. Incorporation of ^{14}C glucose into the tunic of the ascidian *Ciona intestinalis* (Linnaeus). *J. Exp. Zool.* **225:** 187–195.

ROWLEY, A. F. 1981. The blood cells of the sea squirt *Ciona intestinalis:* Morphology, differential counts and *in vitro* phagocytic activity. *J. Invert. Pathol.* **37:** 91–100.

SMITH, M. J. 1970. The blood cells and tunic of the ascidian *Halocynthia aurantium* (Pallas). I. Hematology, tunic morphology, and partition of cells between blood and tunic. *Biol. Bull.* **138:** 354–378.

THOMAS, J. A. 1931. Sur les réations de la tunique d'*Ascidia mentula* Müll., à l'inoculation de *Bacterium tumefaciens* Sm. *C. R. Soc. Biol.* **108:** 694–696.

WRIGHT, R. K. 1981. Urochordates. Pp. 565–626 in *Invertebrate Blood Cells,* Vol. 2, N. A. Ratcliffe and A. F. Rowley, eds. Academic Press, London.

WRIGHT, R. K., AND E. L. COOPER. 1975. Immunological maturation in the tunicate *Ciona intestinalis. Am. Zool.* **15:** 21–27.

WRIGHT, R. K., AND T. H. ERMAK. 1982. Cellular defense systems of the Protochordata. Pp. 283–320 in *The Reticuloendothelial System, a Comprehensive Treatise. Phylogeny and Ontogeny,* Vol. 3, N. Cohen and M. M. Sigel, eds. Plenum Press, New York.

Reference: *Biol. Bull.* **167**: 251–263 (August, 1984)

INFLUENCE OF CHEMICAL COMPOSITION OF ALGAL FOOD SOURCES ON GROWTH OF JUVENILE OYSTERS, *CRASSOSTREA VIRGINICA*

GARY H. WIKFORS, JOSEPH W. TWAROG, JR., AND RAVENNA UKELES

National Marine Fisheries Service, Northeast Fisheries Center, Milford Laboratory, Milford, Connecticut 06460

ABSTRACT

Two algal flagellates, *Dunaliella tertiolecta* Butcher and *Tetraselmis maculata* Butcher, harvested in the stationary phase from a semi-continuous carboy culture apparatus, were analyzed for dry weight, total carbohydrate, total protein, and total lipid. Each species was cultured in three different growth media. The growth response of *D. tertiolecta* was similar in all three formulations but populations of *T. maculata* were considerably limited in the reduced-nutrient medium, X_1. Both algal species cultured in the X_1 medium had significantly greater dry weights and contained more carbohydrate and less protein than cells cultured in the standard formulation (E). A third formulation (N/P), in which all medium components were reduced except nitrate and phosphate, produced algae with reduced carbohydrate and increased protein as compared with E medium. The total lipid content of *D. tertiolecta* was significantly less than that of *T. maculata* regardless of the culture medium.

Algae cultured in the three formulations were fed to juvenile oysters, *Crassostrea virginica*. *T. maculata* was a consistently better food source than *D. tertiolecta*, indicating a probable causal relationship between algal lipid content and oyster growth. Growth of oysters fed algae cultured in X_1 medium was increased as compared with oysters fed algae cultured in E or N/P medium, suggesting a nutritional requirement for relatively more carbohydrate than protein as well. Results indicate that differences in growth media affect the gross chemical composition of algal food sources which alone can account for differences in algal nutritional value to *C. virginica*.

INTRODUCTION

The laboratory or hatchery method of rearing molluscs in which brood stocks of adults are conditioned for spawning and induced to release gametes, and whose fertilized eggs are reared through larval stages to metamorphosis, requires that a suitable source of nutrition be available throughout the life cycle of the species that is being reared. This nutrition is derived most often from the culture of selected species of unicellular algae (see review, Walne, 1964; Ukeles, 1971, 1980; Epifanio, 1982), which are introduced into trays and tanks holding the molluscs. Other methods of obtaining algae have also been used (Glancy, 1965; Castagna, 1975), different sources of nutrition have been explored (Epifanio, 1979), and the development of artificial diets is in progress (Gabbott *et al.*, 1976; Langdon, 1983). From the earliest work in which it was recognized that unicellular algae are food sources for molluscs (Cole, 1936; Bruce *et al.*, 1939) investigators have queried why some species of algae are better food sources for molluscs than others. Certain factors related to the algal

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cell have been suggested as explanations, *e.g.*, cell size, cell wall composition, digestibility, the presence or absence of toxic metabolites, and chemical composition of the algal cell. None of these characteristics alone has offered an entirely satisfactory explanation. Although it seems that gross chemical composition (protein, carbohydrate, lipid) of different algae should be highly significant in affecting molluscan growth, research on this subject has yielded inconclusive results (see review, Webb and Chu, 1982).

The research reported here describes the manner in which the gross chemical composition of two algal species of close taxonomic standing can be induced to vary and how the chemistry of the algal food source influences the growth response of juvenile oysters, *Crassostrea virginica.*

Materials and Methods

Algal culture and population determination

Dunaliella tertiolecta Butcher and *Tetraselmis maculata* Butcher, obtained from the Milford algal culture collection, were examined. These species, cultured axenically in an enriched sea water growth medium, E, have been in the collection for many years, and strains of these phytoplankters have also been maintained in a reduced-nutrient enriched sea water medium, X_1, for several years. Strains cultured in a third formulation in which all medium components except nitrate and phosphate were reduced, N/P, were developed during the course of these experiments. Inocula for the growth curves reported in this study had been subcultured routinely in N/P for over one year. Medium formulations are shown in Table I.

Algae were cultured in the following types of Pyrex glassware: 20 × 150-mm screw-capped test tubes (with the liners removed) that were matched and calibrated for use as cuvettes in growth experiments; 125- and 500-ml Erlenmeyer flasks; and Fernbach flasks fitted with siphons and filling bell attachments to inoculate semi-continuous 18-liter carboy cultures (Ukeles, 1973).

Table I

Formulations for algal growth media; final concentrations for 1 liter

Component	Medium		
	X_1	N/P	E
Sea Water*	500 ml	500 ml	500 ml
$NaNO_3$	77.5 mg	300 mg	300 mg
KH_2PO_4	5.0 mg	20 mg	20 mg
NaFe Sequestrene	0.5 mg	0.5 mg	5 mg
THAM**	0.5 gm	0.5 gm	1.0 gm
Vitamin B_{12}	0.8 μg	0.8 μg	3.0 μg
Thiamin HCl	0.08 mg	0.08 mg	0.3 mg
$CuSO_4 \cdot 5H_2O$	4.9 ng	4.9 ng	9.8 ng
$ZnSO_4 \cdot 7H_2O$	11 ng	11 ng	22 ng
$CoCl_2 \cdot 6H_2O$	6.5 ng	6.5 ng	13 ng
$MnCl_2 \cdot 4H_2O$	90 ng	90 ng	180 ng
$NaMoO_4 \cdot 2H_2O$	3.1 ng	3.1 ng	6.2 ng

Adjust pH to 8.0 and bring to volume of 1 liter with double glass-distilled water.

* Salinity 27–28‰.

** Tris(hydroxymethyl)aminomethane.

Media were steam-sterilized in a Castle autoclave for 20 minutes (40 minutes for carboy media) at 15 psi pressure. Test tube and flask cultures were incubated in a GPI Model RI Incubator/Growth Chamber at 20°C, illuminated to about 300 ftC with cool-white fluorescent lights on a 12:12-h light/dark cycle. Carboys received constant illumination (300 ftC) at 20°C ± 1°C.

Culture densities for constructing growth curves were determined turbidimetrically with a Bausch & Lomb Spectronic 20 Spectrophotometer-colorimeter. Algal populations were also determined by microscopic counts in an Improved Neubauer Hemacytometer (Bright Line). Carboy cultures which had been harvested daily and replenished weekly with sterilized media for four weeks were selected for analyses. Samples of algae taken five days after the most recent addition of medium were subjected to dry weight determinations according to the method of Epifanio and Ewart (1977) and chemical analyses, as described in the next section.

Analyses of algal chemical constituents

Analyses of total protein were conducted using a heated biuret-Folin (HBF) procedure modified from Dorsey *et al.* (1977, 1978), a method which provided increased sensitivity and a more stable end point as compared with the well-known method of Lowry *et al.* (1951). Cells were collected on a 25-mm glass fiber filter, and protein was extracted with appropriate reagents at 100°C for 100 minutes. Modifications from the published procedure primarily involved the concentrations and final volumes of reagents in the reaction tube (Table II). Sample reactions were read in a Beckman DB GT Grating Spectrophotometer. Protein nitrogen values were determined by interpolation from a standard curve obtained with prepared solutions of bovine serum albumin. Total protein was then calculated using a conversion factor of 6.25 generally accepted for most marine algal species (Dorsey *et al.*, 1978).

Carbohydrate determinations were made using a phenol-sulfuric acid method for analysis of algae reported by Kochert (1978) based upon procedures developed by Dubois *et al.* (1956). Algal culture samples were collected and washed with sterile isotonic NaCl by repeated cold centrifugation in an IEC Model Pr-2 centrifuge. Cells to be assayed were then homogenized in ethanol as in Myklestad and Haug (1972); this method was modified by using a Sonicor 50W ultrasonic bath. Prepared glucose solutions were assayed to construct a standard curve as recommended by Marshall and Orr (1962).

TABLE II

Preparation of solutions for heated biuret-Folin protein assay

Reagent	Concentration of stock solution	ml for intermediate working solution	ml for 100 ml final solution*	w/v concentration upon addition to reaction tube*
Na_2CO_3	20 g/100 ml	—	10 ml	2%
NaOH	40 g/1000 ml	—	10 ml	0.4%
NaKTartrate	20 g/100 ml	2 ml/10 ml combine**	1 ml	0.04%
$CuSO_4 \cdot 5H_2O$	5 g/100 ml	2 ml/10 ml		0.01%

* This corresponds with "reagent f" of Dorsey *et al.* (1977).

** To keep $CuSO_4 \cdot 5H_2O$ from precipitating, it is necessary to dilute NaKTartrate to about 7 ml before adding Cu solution, mix thoroughly, and then bring to final volume of 10 ml with twice-distilled water.

Determinations of total lipid were conducted using the method of Mukerjee (1956) as adapted by Strickland and Parsons (1968). Cells for lipid analyses were collected, washed, and homogenized as in the carbohydrate procedure described above. A precipitate of algal material obtained by evaporating the ethanol from the homogenate was assayed, and extinctions were corrected with appropriate stearic acid standard and blank determinations.

Oyster feeding experiments

Juvenile oysters, *Crassostrea virginica,* were grown in molluscan rearing chambers, described in Ukeles and Wikfors (1982), which provided a constant flow of filtered, UV-irradiated sea water with temperature adjusted to 26°C. Each day, sea water flow was interrupted for four hours during which oysters were permitted to feed upon algal food suspensions introduced into the chambers. Daily rations of algal food cultures harvested from carboys were equilibrated to the same cytoplasmic volume of 0.6 ml packed cells per chamber as determined by centrifugation in modified Hopkins tubes (Ukeles, 1973). Resulting daily nutritional inputs in terms of algal cell number, dry weight, protein, carbohydrate, and lipid were calculated for each algal food source. Differences between means of replicate chemical analyses and daily nutritional inputs were tested with a Z test ($\alpha = 0.05$).

Each molluscan rearing chamber contained 50 juvenile oysters of similar initial size. Brood stocks of adult oysters obtained from a commercial landing at New Haven, Connecticut, were conditioned in the laboratory for spawning and induced to release gametes by warm-water stimulation (Loosanoff and Davis, 1963). Fertilized eggs were reared through setting in filtered sea water on a diet of cultured algae. Young juvenile oysters thus obtained were utilized in these experiments. Growth of oysters was determined weekly by weighing pooled groups of 50 live oysters from each chamber on a Sartorius top loading balance. Mean live weight per oyster was calculated for each population and plotted *versus* time in weeks.

RESULTS

Algal growth populations

Population growth of *D. tertiolecta* cultured in E, N/P, and X_1 media was nearly identical (Fig. 1). The effects of various NO_3/PO_4 ratios were investigated in a factorial design experiment where 16 combinations of $NaNO_3$ (77.5, 150, 300, and 350 mg/l) and KH_2PO_4 (5, 10, 20, and 25 mg/l) were included in the X_1 basal medium. Population growth of *D. tertiolecta* was similar over this range of concentrations; representative growth curves in nitrate/phosphate ratios of 77.5/5, 300/5, 77.5/20, and 300/20 mg/l are shown in Figure 2. Increased concentrations of chelated iron (up to 1.5 mg/l) and vitamins (B_{12} up to 6.4 μg/l and thiamine·HCl up to 0.64 mg/l) were also tested in an X_1 base formulation for possible growth-promoting effects upon *D. tertiolecta.* Again, no differences in the growth of *D. tertiolecta* were detected. Clearly, population growth of *D. tertiolecta* is not affected by the reduction of major medium components from E to X_1 levels.

In contrast, *Tetraselmis maculata* demonstrated a considerable reduction of growth in the X_1 formulation (Fig. 3). This difference did not appear as a diminished rate of logarithmic growth but, rather, as a decrease in the maximum population as compared with E medium. The addition of nitrate and phosphate to X_1 medium in concentrations equivalent to those in the E medium (= N/P) produced a population density equivalent to that obtained in E medium (Fig. 3). Increasing the vitamin

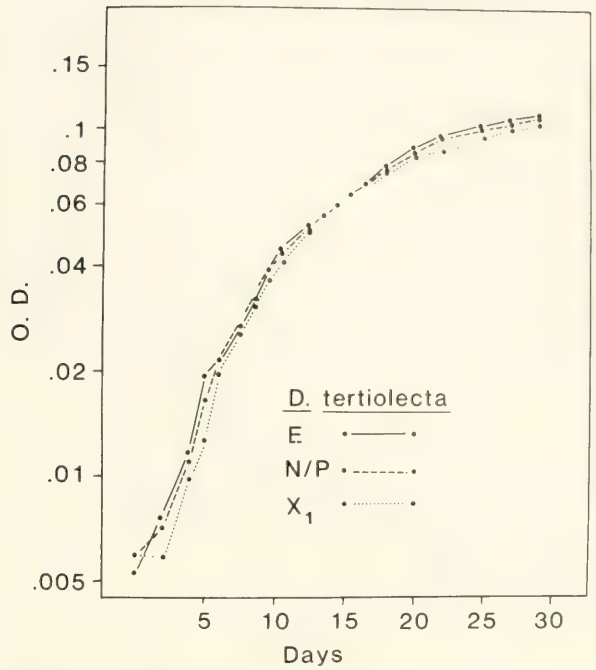

FIGURE 1. Growth curves of *Dunaliella tertiolecta* populations in three enriched sea water media: E, N/P, and X_1.

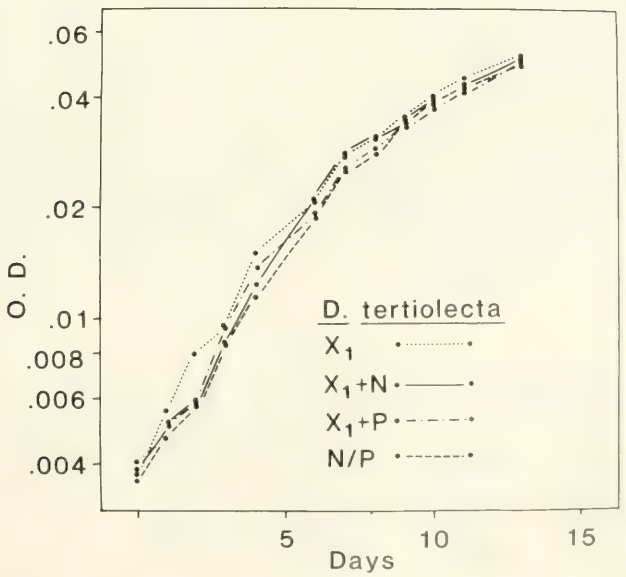

FIGURE 2. Growth curves of *Dunaliella tertiolecta* populations in X_1 basal medium, X_1 medium with increased $NaNO_3$ (total 200 μg/l), X_1 medium with increased KH_2PO_4 (total 20 mg/l), and N/P medium.

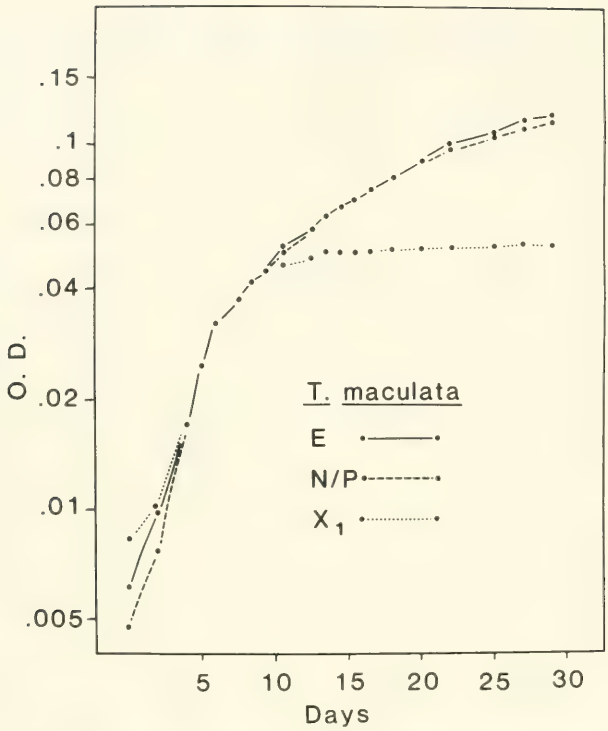

FIGURE 3. Growth curves of *Tetraselmis maculata* populations in three enriched sea water media: E, N/P, and X_1.

concentrations in X_1 medium up to 6.4 μg/l for B_{12} and 0.64 mg/l for thiamine · HCl did not affect any increase in *T. maculata* growth.

Dry weights of 10^6 cells of *D. tertiolecta* and *T. maculata* differed significantly ($\alpha = 0.05$) when algae were cultured in the three formulations. *D. tertiolecta* cells cultured in X_1 medium had dry weights that were larger than those from E or N/P; the dry weights of cells from the latter two were not different statistically (Table III). Similarly, *T. maculata* cells had greater dry weights when cultured in X_1 medium than in E or N/P (Table III).

TABLE III

Gross chemical composition of algal cells

Species	Medium	Dry weight μg/10^6 cells	% Total protein	% Total carbohydrate	% Total lipid
Dunaliella tertiolecta	X_1	89.93	17.2	56.0	2.4
	E	78.26	39.4	31.1	2.0
	N/P	77.69	57.4	23.2	1.5
Tetraselmis maculata	X_1	119.5	15.6	36.5	15.3
	E	110.9	31.0	23.9	8.3
	N/P	114.7	37.9	13.5	9.2

Algal chemical composition

Although population growth of *D. tertiolecta* was not affected by the reduction of nutrients to concentrations contained in X_1 or N/P formulations, the chemical compositions of cells cultured in E, N/P, and X_1 media differed statistically. Differences in percent total carbohydrate between *D. tertiolecta* cells cultured in the three media were significant ($\alpha = 0.05$) $X_1 > E > N/P$, and the same observation was made for *T. maculata* (Table III). Conversely percent total protein for each species cultured in the three media showed the opposite relationship ($\alpha = 0.05$) $N/P > E > X_1$ (Table III). The percentage of the dry weight accounted for by the lipid fraction was significantly ($\alpha = 0.05$) larger in *T. maculata* than in *D. tertiolecta* regardless of growth medium. Lipid content of both algae seems to be somewhat increased in X_1 over E and N/P media, but these differences could not be tested statistically.

Oyster feeding experiments

Daily rations of algal food sources were adjusted to provide identical cytoplasmic volumes as previously described. Because algal cells cultured in different media were shown to have different dry weights and chemical compositions, it was necessary to compare daily rations for all algal food cultures in terms of number of cells, dry weight, and the chemical components in mg fed per day (Table IV). The outstanding difference in daily ration between *D. tertiolecta* and *T. maculata* involved the lipid component of the diet: oysters fed *T. maculata* received significantly more lipid than those fed *D. tertiolecta.* Daily rations of protein varied from 24.2 mg in X_1 to 68.2 mg in N/P for *D. tertiolecta* and from 23.9 mg in X_1 to 81.8 mg in N/P for *T. maculata.* Total carbohydrate in the algal diets ranged from 27.6 mg in N/P to 78.6 mg in X_1 for *D. tertiolecta* and from 33.4 mg in N/P to 55.9 mg in X_1 for *T. maculata.* Dry weights of 0.6 ml packed cells were higher for *T. maculata* than for *D. tertiolecta* with the *T. maculata* N/P diet having the largest dry weight, 248 mg, and *D. tertiolecta* in E medium having the smallest, 113 mg. Numbers of cells contained in the daily rations were very similar for the three *D. tertiolecta* diets ranging only from 1.44–1.56 $\times 10^9$ cells. The *T. maculata* diets showed more variation with a range of 1.28–2.16 $\times 10^9$ cells (in X_1 and N/P, respectively).

Growth responses of juvenile oysters to *D. tertiolecta* or *T. maculata* cultured in E, N/P, or X_1 medium are shown in Figure 4. Oysters exhibited increased growth when fed *T. maculata* as compared with *D. tertiolecta* regardless of the medium in which the cells were cultured ($\alpha = 0.05$). After six weeks of observation, oysters fed either alga cultured in X_1 medium were larger than oysters fed algae cultured in E

TABLE IV

Composition of 0.6 ml packed cells for six algal diets fed to oyster populations daily

Algal species	Culture medium	Number of cells	Dry weight	Total carbohydrate	Total protein	Total lipid
Dunaliella tertiolecta	X_1	1.56×10^9	140 mg	78.6 mg	24.2 mg	3.34 mg
	E	1.44×10^9	113 mg	35.1 mg	44.4 mg	2.22 mg
	N/P	1.53×10^9	119 mg	27.6 mg	68.2 mg	1.74 mg
Tetraselmis maculata	X_1	1.28×10^9	153 mg	55.9 mg	23.9 mg	23.4 mg
	E	1.94×10^9	215 mg	51.4 mg	66.8 mg	18.0 mg
	N/P	2.16×10^9	248 mg	33.4 mg	81.8 mg	22.8 mg

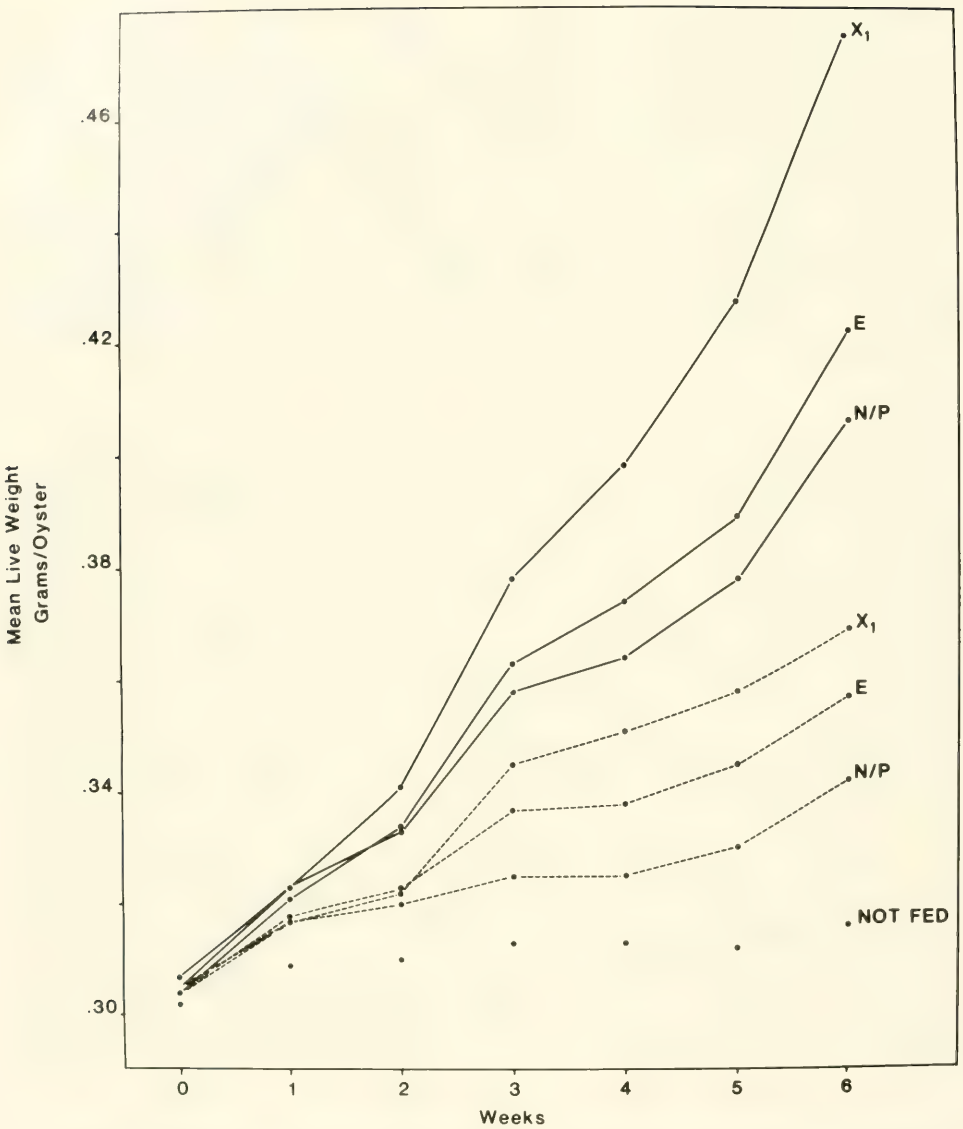

FIGURE 4. Growth of juvenile oysters, *Crassostrea virginica,* fed *Dunaliella tertiolecta* (dashed line), and *Tetraselmis maculata* (solid line), each cultured in three different medium formulations: E, X_1, and N/P.

medium which, in turn, had grown more than oysters fed algae cultured in the N/P formulation. This relationship was consistent for both *T. maculata* and *D. tertiolecta;* however, statistical evaluation was difficult. Although data points shown in Figure 4 represent an average weight for 50 oysters from one chamber, these 50 were weighed as a pooled group; the small size and large numbers of the oysters rendered weighing individuals impractical. Over six weeks, calculated differences between mean weekly growth rates of oysters fed X_1, E, or N/P cultures were not significant ($\alpha = 0.05$) with the exception of *T. maculata* in which $X_1 >$ N/P. This is not

surprising considering the somewhat hyperbolic shapes of the growth curves. Nevertheless, the consistency of the growth curves suggests that differences in growth of oysters fed either alga cultured in X_1, E, or N/P represent a genuine response to the different diets.

Discussion

In the present investigation, *T. maculata* and *D. tertiolecta* were compared as food sources for juvenile *C. virginica.* These algae are similar in that both are chlorophyte flagellates, ovoid or elliptical in shape with yellow-green chromatophores, and are of similar size (cell length of *D. tertiolecta* ranges from 6.4–11.7 μm and *T. maculata* from 6.2–13.8 μm); the main difference is the presence of a medium rigid cell wall in *T. maculata* and a thin hyaline periplast in *D. tertiolecta* (Butcher, 1959). The algae were cultured in three different media formulations which resulted in differences in cell chemistry. These findings afforded a unique opportunity for evaluation of the significance of algal chemical composition in the nutrition of the filter-feeding bivalve, *Crassostrea virginica.* The highly controlled conditions provided by the use of axenic cultures and the chambers designed for rearing the bivalves served to eliminate many of the unknown variables that can affect data collected in feeding studies conducted under less rigorous experimental conditions.

D. tertiolecta tolerated a wide variation in nutrient enrichment of its growth medium; population growth showed little variation between the standard E formulation, the much reduced X_1, and the N/P formulation. In an earlier study it was also observed that two algal species used as molluscan food sources, *Monochrysis lutheri* Droop (= *Pavlova lutheri* comb. nov. Green) and *Phaeodactylum tricornutum* Bohlin can tolerate a substantial (50%) reduction of phosphate and nitrate concentrations in the growth medium (Ukeles, 1977). *T. maculata,* in contrast, responded to the low NO_3 and PO_4 concentrations of X_1 medium with a reduced population; its needs for N and P were evidently higher than for *D. tertiolecta.*

Although the growth of *D. tertiolecta* populations was unaffected by variations in the three nutrient formulations of the growth medium, the chemical composition varied considerably. Stationary phase cells cultured in E medium and, more so, in X_1 medium accumulated more carbohydrate than in the N/P medium. The composition of cells in N/P medium was higher in protein. The low carbohydrate and high protein contents found in N/P medium were similar to those reported for log phase *D. salina* cultured in an artificial sea water medium (Parsons *et al.,* 1961).

The protein concentration of both flagellates is lowest in the X_1 medium and, in fact, the concentrations of protein in the three growth media are directly reversed to those of carbohydrates. Algae seem to have exhausted nitrogen for protein synthesis in the X_1 medium and shunted fixed carbon to carbohydrate synthesis without suffering a reduction in final population as measured by optical density. This observation agrees with previous studies reporting that stationary phase algal cells, which accumulate carbohydrates (Handa, 1969; Myklestad and Haug, 1972; Chu *et al.,* 1982a; Loos and Meindl, 1982), are actually doing so in response to nitrogen depletion in the medium (Antia *et al.,* 1963; Werner, 1970; Hobson and Pariser, 1971). Stationary phase cultures of *T. maculata* from N/P medium, similar to *D. tertiolecta,* contained a high protein, low carbohydrate composition, agreeing with the results reported for analysis of log phase cultures of *T. maculata* by Parsons *et al.* (1961).

D. tertiolecta, as an oyster food, did not increase in nutritional value relative to *T. maculata* when cellular protein or carbohydrate was increased. Similarly, *T. maculata* did not diminish in value relative to *D. tertiolecta* when protein or carbohydrate

was decreased in the different growth media. Additional evidence that the amount of protein or carbohydrate fed per day does not limit the growth of juvenile *C. virginica* lies in the observation that the *D. tertiolecta* X_1 diet contained more carbohydrate than any *T. maculata* diet, and the *D. tertiolecta* N/P diet contained more protein than the *T. maculata* X_1 diet (Table IV). In no case was growth of oysters fed *D. tertiolecta* greater than that of oysters fed *T. maculata.* However, the lipid contents of *T. maculata* in all three diets were greater than those of *D. tertiolecta* by a factor of 10 (Table IV). Thus, there is strong evidence that total lipid, or a component of the lipid fraction, in *T. maculata* cells accounts for this alga's greater food value as compared with *D. tertiolecta.* Evidence that lipid content of a food source is critical in determining the growth response of oysters has been suggested in feeding studies with juvenile *C. virginica* offered different artificial diets (Castell and Trider, 1974), and the importance of lipids in the diet for larval bivalves has been well-documented (Millar and Scott, 1967; Helm *et al.,* 1973; Holland and Spencer, 1973; Holland, 1978; Chu and Dupuy, 1980; Chu *et al.,* 1982b). Components found in lipids extracted from algal cells showed that certain fatty acids, particularly those of the 6 *w* 3 group, are required by oysters (Langdon and Waldock, 1981; Langdon, 1982). The former study showed that a diet of cultured *D. tertiolecta* cells, fortified with encapsulated 22:6 *w* 3 fatty acid, produced better growth of *C. gigas* juveniles than did *D. tertiolecta* alone. It is possible, then, that *T. maculata* contains a required lipid component, perhaps a fatty acid, in large amounts as compared with *D. tertiolecta.* The greater nutritional value of *T. maculata* than *D. tertiolecta* is clearly correlated with the greater lipid concentrations in *T. maculata.*

It is noteworthy that both species cultured in the X_1 formulation in which the carbohydrate content was higher than the other two growth media offered the best oyster nutrition of the three media tested. Several investigators have implicated carbohydrates, particularly glucose, as being important in oyster nutrition (Gillespie *et al.,* 1964; Haven, 1965; Dunathan *et al.,* 1969). Flaak and Epifanio (1978), using *Thalassiosira pseudonana* as a food source, concluded that the growth of the oyster, *C. virginica,* was more rapid when algae were richer in carbohydrate than protein.

It has been known for some time that the chemical composition of freshwater algae can be affected by variation in environmental factors (Spoehr and Milner, 1948; Taub and Dollar, 1965; Saddler and Taub, 1972). Marine species, particularly diatoms, also have been shown to undergo changes in composition with alterations of environmental conditions. Observations of a phytoplankton bloom induced in a plastic sphere showed that with nitrogen depletion in the medium a significant change in the carbohydrate and protein composition of cells occurred (Antia *et al.,* 1963). A decrease in N/C ratio as a result of nitrogen deficiency has been observed in *Skeletonema costatum* (Holm-Hansen *et al.,* 1968), *Cyclotella nana,* and *Thalassiosira fluviatilis* (Hobson and Pariser, 1971). Myklestad and Haug (1972) found that with a depletion in nitrate there was a change in the protein/carbohydrate ratio in *Chaetoceros affinis.* Protein, carbohydrate, and lipid composition of two strains of *Phaeodactylum tricornutum* were found to vary in light and dark periods (Terry *et al.,* 1983) and it was also observed that temperature and light conditions will vary the composition of *Thalassiosira allenii* (Redalje and Laws, 1983). The present study has shown that the chemical composition of two closely related flagellates can depend upon the availability of nutrients in the growth medium.

The extreme variation in chemical composition of these algal species that was shown to be dependent upon nutrient availability poses a number of questions pertinent to ecological monitoring, as well as to the practical considerations of providing a source of nutrition for artificially reared filter-feeding species. High primary productivity

of marine and estuarine waters, as measured by algal cell numbers, chlorophyll content of sea water, dry weight of biomass, fixed carbon, or other methods currently employed by environmental biologists and oceanographers, may not necessarily reflect accurately the ability of these waters to support large populations in higher trophic levels. Predictions of harvests of commercially valuable species based upon "primary productivity" measurements alone could be overestimated if phytoplankton populations are deficient in some nutrient essential to the grazing species. Similarly, the nutritional values of cultured algae used to rear molluscan or other species in the laboratory or commercial hatchery could vary considerably depending upon algal species, medium formulations, growth phase of algae at harvest, or temperature and light conditions of culture.

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LITERATURE CITED

ANTIA, N. J., C. D. MCALLISTER, T. R. PARSONS, K. STEPHENS, AND J. D. H. STRICKLAND. 1963. Further measurements of primary production using a large-volume plastic sphere. *Limnol. Oceanogr.* **8**: 166–183.

BRUCE, J. R., M. KNIGHT, AND M. W. PARKE. 1939. The rearing of oyster larvae on an algal diet. *J. Mar. Biol. Assoc. U. K.* **24**: 337–374.

BUTCHER, R. W. 1959. An introductory account of the smaller algae of British coastal waters. Part I: Introduction and chlorophyceae. *Min. Agric. Fish. Food Fishery Invest. Ser.* **IV**: 1–74.

CASTAGNA, M. 1975. Culture of the bay scallop, *Argopecten irradians*, in Virginia. *Mar. Fish. Rev.* **37**: 19–24.

CASTELL, J. D., AND D. J. TRIDER. 1974. Preliminary feeding trials using artificial diets to study the nutritional requirements of oysters (*Crassostrea virginica*). *J. Fish. Res. Board Can.* **31**: 95–99.

CHU, F.-L. E., AND J. L. DUPUY. 1980. The fatty acid composition of three unicellular algal species used as food sources for larvae of the American oyster (*Crassostrea virginica*). *Lipids* **15**: 356–364.

CHU, F.-L. E., J. L. DUPUY, AND K. L. WEBB. 1982a. Polysaccharide composition of five algal species used as food for larvae of the American oyster, *Crassostrea virginica*. *Aquaculture* **29**: 241–252.

CHU, F.-L. E., K. L. WEBB, D. HEPWORTH, AND M. ROBERTS. 1982b. The acceptability and digestibility of microcapsules by larvae of *Crassostrea virginica*. *J. Shellfish Res.* **2**: 29–34.

COLE, H. A. 1936. Experiments in the breeding of oysters (*Ostrea edulis*) in tanks, with special reference to the food of the larva and spat. *Min. Agric. Fish. Fishery Invest. Ser. II*, Vol. **XV**: 1–24.

DORSEY, T. E., P. W. MCDONALD, AND O. A. ROELS. 1977. A heated biuret-Folin protein assay which gives equal absorbance with different proteins. *Anal. Biochem.* **78**: 156–164.

DORSEY, T. E., P. MCDONALD, AND O. A. ROELS. 1978. Measurements of phytoplankton-protein content with the heated biuret-Folin assay. *J. Phycol.* **14**: 167–171.

DUBOIS, M., K. A. GILLES, J. K. HAMILTON, P. A. REBERS, AND F. SMITH. 1956. Colorimetric method for determination of sugars and related substances. *Anal. Chem.* **18**: 350–356.

DUNATHAN, J. P., R. M. INGLE, AND W. K. HAVENS, JR. 1969. Effects of artificial foods upon oyster fattening with potential commercial applications. *State of Florida Dept. Natural Resources, Tech. Ser. No. 58, Div. Mar. Resources*, St. Petersburg. 39 pp.

EPIFANIO, C. E. 1979. Comparison of yeast and algal diets for bivalve molluscs. *Aquaculture* **16**: 187–192.

EPIFANIO, C. E. 1982. Phytoplankton and yeast as foods for juvenile bivalves: A review of research at the University of Delaware. Pp. 292–304 in *Proc. Second International Conference on Aquaculture Nutrition: Biochemical and Physiological Approaches to Shellfish Nutrition*, G. D. Pruder, C. Langdon, and D. Conklin, eds. Louisiana State University, Baton Rouge.

EPIFANIO, C. E., AND J. EWART. 1977. Maximum ration of four algal diets for the oyster *Crassostrea virginica* Gmelin. *Aquaculture* **11**: 13–29.

FLAAK, A. R., AND C. E. EPIFANIO. 1978. Dietary protein levels and growth of the oyster *Crassostrea virginica*. *Mar. Biol.* **45**: 157–163.

GABBOTT, P. A., D. A. JONES, AND D. H. NICHOLS. 1976. Studies on the design and acceptability of micro-

encapsulated diets for marine particle feeders. II. Bivalve molluscs. Pp. 127–141 in *Tenth European Symposium on Marine Biology* (Ostend, Belgium), G. Persoone and E. Jaspers, eds. Universa Press, Wetteren.

GILLESPIE, L., R. M. INGLE, AND W. K. HAVENS. 1964. Glucose nutrition and longevity in oysters. *Q. J. Florida Acad. Sci.* **27:** 279–288.

GLANCY, J. B. 1965. Method of raising shellfish seed in a simulated habitat. *U. S. Patent Office.* Patent No. 3,196,833.

HANDA, N. 1969. Carbohydrate metabolism in the marine diatom, *Skeletonema costatum. Mar. Biol.* **4:** 208–214.

HAVEN, D. S. 1965. Supplemental feeding of oysters with starch. *Chesapeake Sci.* **6:** 43–51.

HELM, M. M., D. L. HOLLAND, AND R. R. STEPHENSON. 1973. The effect of supplementary algal feeding of a hatchery breeding stock of *Ostrea edulis* L. on larval vigour. *J. Mar. Biol. Assoc. U. K.* **53:** 673–684.

HOBSON, L. A., AND R. J. PARISER. 1971. The effect of inorganic nitrogen on macromolecular synthesis by *Thalassiosira fluviatilis* Hustedt and *Cyclotella nana* Hustedt grown in batch culture. *J. Exp. Mar. Biol. Ecol.* **6:** 71–78.

HOLLAND, D. L. 1978. Lipid reserves and energy metabolism in larvae of benthic marine invertebrates. Pp. 85–123 in *Biochemical and Biophysical Perspectives in Marine Biology,* 4, D. C. Malins and J. R. Sargent, eds. Academic Press, London.

HOLLAND, D. L., AND B. E. SPENCER. 1973. Biochemical changes in fed and starved oysters, *Ostrea edulis* L. during larval development, metamorphosis and early spat growth. *J. Mar. Biol. Assoc. U. K.* **53:** 287–298.

HOLM-HANSEN, O., W. H. SUTCLIFFE, JR., AND J. SHARP. 1968. Measurement of deoxyribonucleic acid in the ocean and its ecological significance. *Limnol. Oceanogr.* **13:** 507–514.

KOCHERT, G. 1978. Carbohydrate determination by the phenol-sulfuric acid method. Pp. 95–97 in *Handbook of Phycological Methods—Physiological and Biochemical Methods,* J. A. Hellebust and J. S. Craigie, eds. Cambridge University Press, New York.

LANGDON, C. J. 1982. New techniques and their application to studies of bivalve nutrition. Pp. 305–320 in *Proc. Second International Conference on Aquaculture Nutrition: Biochemical and Physiological Approaches to Shellfish Nutrition,* G. D. Pruder, C. Langdon, and D. Conklin, eds. Louisiana State University, Baton Rouge.

LANGDON, C. J. 1983. Growth studies with bacteria-free oyster (*Crassostrea gigas*) larvae fed on semi-defined artificial diets. *Biol. Bull.* **164:** 227–235.

LANGDON, C. J., AND M. J. WALDOCK. 1981. The effect of algal and artificial diets on the growth and fatty acid composition of *Crassostrea gigas* spat. *J. Mar. Biol. Assoc. U. K.* **61:** 431–448.

LOOS, E., AND D. MEINDL. 1982. Composition of the cell wall of *Chlorella fusca. Planta* **156:** 270–273.

LOOSANOFF, V. L., AND H. C. DAVIS. 1963. Rearing of bivalve mollusks. Pp. 1–136 in *Advances in Marine Biology, I,* F. S. Russell, ed. Academic Press, London.

LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, AND R. J. RANDALL. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.

MARSHALL, S. M., AND A. P. ORR. 1962. Carbohydrate as a measure of phytoplankton. *J. Mar. Biol. Assoc. U. K.* **42:** 511–519.

MILLAR, R. H., AND J. M. SCOTT. 1967. The larvae of the oyster *Ostrea edulis* during starvation. *J. Mar. Biol. Assoc. U. K.* **47:** 475–484.

MUKERJEE, P. 1956. Use of ionic dyes in the analysis of ionic surfactants and other ionic organic compounds. *Anal. Chem.* **28:** 870–873.

MYKLESTAD, S., AND A. HAUG. 1972. Production of carbohydrates by the marine diatom *Chaetoceros affinis* var. *willei* (Gran) Hustedt. I. Effect of the concentration of nutrients in the culture medium. *J. Exp. Mar. Biol. Ecol.* **9:** 125–136.

PARSONS, T. R., K. STEPHENS, AND J. D. H. STRICKLAND. 1961. On the chemical composition of eleven species of marine phytoplankters. *J. Fish. Res. Board Can.* **18:** 1001–1016.

REDALJE, D. G., AND E. A. LAWS. 1983. The effects of environmental factors on growth and the chemical and biochemical composition of marine diatoms. I. Light and temperature effects. *J. Exp. Mar. Biol. Ecol.* **68:** 59–79.

SADDLER, J. B., AND F. B. TAUB. 1972. Chemical variability of algal shellfish feeds. *Proc. Natl. Shellfish. Assoc.* **62:** 6–7.

SPOEHR, H. A., AND H. W. MILNER. 1948. The chemical composition of *Chlorella;* effect of environmental conditions. *Plant Physiol.* **24:** 120–149.

STRICKLAND, J. D. H., AND T. R. PARSONS. 1968. A practical handbook of seawater analysis. *Bull. Fish. Res. Board Can.* **167:** 1–310.

TAUB, F. B., AND A. M. DOLLAR. 1965. Control of protein level of algae, *Chlorella. J. Food Sci.* **30:** 359–364.

TERRY, K. L., J. HIRATA, AND E. A. LAWS. 1983. Light-limited growth of two strains of the marine diatom *Phaeodactylum tricornutum* Bohlin: Chemical composition, carbon partitioning and the diel periodicity of physiological processes. *J. Exp. Mar. Biol. Ecol.* **68**: 209–227.

UKELES, R. 1971. Nutritional requirements in shellfish culture. Pp. 43–64 in *Proc. Conference on Artificial Propagation of Commercially Valuable Shellfish*, K. S. Price, Jr. and D. L. Maurer, eds. University of Delaware, Newark.

UKELES, R. 1973. Continuous culture—a method for the production of unicellular algal foods. Pp. 233–254 in *Handbook of Phycological Methods—Culture Methods and Growth Measurements*, J. Stein, ed. Cambridge University Press, London.

UKELES, R. 1977. Culture of algae for feeding larval and juvenile molluscs in controlled aquaculture. Third Meeting of the I.C.E.S. Working Group on Mariculture, Brest, France, May 10–13. *Actes de Colloques du C.N.E.X.O.* **4**: 361–370.

UKELES, R. 1980. American experience in the mass culture of micro-algae for feeding larvae of the American oyster, *Crassostrea virginica*. Pp. 287–306 in *Algae Biomass, Production and Use*, G. Shelef and C. J. Soeder, eds. Elsevier/North-Holland Biomedical Press, Amsterdam.

UKELES, R., AND G. H. WIKFORS. 1982. Design, construction, and operation of a rearing chamber for spat of *Crassostrea virginica* (Gmelin). *J. Shellfish Res.* **2**: 35–39.

WALNE, P. R. 1964. The culture of marine bivalve larvae. Pp. 197–210 in *Physiology of Mollusca, I*, K. M. Wilbur and C. M. Yonge, eds. Academic Press, New York.

WEBB, K. L., AND F.-L. E. CHU. 1982. Phytoplankton as a food source for bivalve larvae. Pp. 272–291 in *Proc. Second International Conference on Aquaculture Nutrition: Biochemical and Physiological Approaches to Shellfish Nutrition*, G. D. Pruder, C. Langdon, and D. Conklin, eds. Louisiana State University, Baton Rouge.

WERNER, D. 1970. Productivity studies on diatom cultures. *Helgol. Wiss. Meeresunters.* **20**: 97–103.

Reference: *Biol. Bull.* **167**: 264–269. (August, 1984)

SIDE-SCAN SONAR RECORDS AND DIVER OBSERVATIONS OF THE GRAY WHALE (*ESCHRICHTIUS ROBUSTUS*) FEEDING GROUNDS

JOHN S. OLIVER AND RIKK G. KVITEK

Moss Landing Marine Laboratories, Moss Landing, California 95039

ABSTRACT

Gray whales (*Eschrichtius robustus*) excavate infaunal invertebrates and sediment by suction, producing many large depressions in the sea floor. Diver observations indicate that side-scan sonar provides accurate estimates of the size of feeding excavations and the area of bottom covered by excavations (>30% of the bottom). Although side scan does not detect some excavations because of small size (particularly <3 m^2) or their orientation with respect to the side-scan track, it gives a quantitative impression of the relative intensity of bottom disturbance by whales. This disturbance is directly related to habitat and prey utilization by whales.

INTRODUCTION

Gray whales (*Eschrichtius robustus*) extensively excavate the sea floor while feeding on benthic invertebrates (Oliver *et al.*, 1983b, 1984). The major prey are amphipod crustaceans living in bottom sediments (Rice and Wolman, 1971; Zimushko and Ivashin, 1980; Bogoslovskaya *et al.*, 1981). Field observations show that both infaunal prey and sediments are extracted by suction (Oliver *et al.*, 1983b, 1984). (See Ray and Schevill, 1974 for "laboratory" observations of suction.) Sediment is expelled through the baleen. Excavation size and shape are highly variable. Gray whales rework single or multiple feeding excavations into much larger and complex features. Distinct excavations range in diameter from less than 1 m to over 20 m. While some feeding excavations are shallow surface sucks (3–10 cm deep), many excavations are 15–30 cm deep, and some are over 40 cm deep (Oliver *et al.*, 1983b, 1984).

The feeding excavations of gray whales are detected by side-scan sonar (Johnson *et al.*, 1983) and are easily distinguished from other depressions in the sea floor made by walrus (Oliver *et al.*, 1983a), ice gouging (Reimnitz *et al.*, 1977; Thor and Nelson, 1981), and gas craters (Nelson *et al.*, 1979). However, does side-scan sonar accurately represent feeding excavations and provide a useful relative impression of feeding disturbance? We answer this question by comparing side-scan records and diver observations of a highly accessible feeding ground along Vancouver Island, where prey communities and feeding records are remarkably similar to the primary feeding ground in the Bering Sea (Oliver *et al.*, 1984).

MATERIALS AND METHODS

The major study area was in Pachena Bay on the west coast of Vancouver Island, British Columbia. Gray whales fed in the bay during the spring and summer on a dense community of tube-dwelling *Ampelisca* amphipods (Oliver *et al.*, 1984). Two permanent underwater stations were established in Pachena Bay to compare side-scan records with diver observations from 16 July to 15 August 1983. The two stations represented areas with relatively few and many excavations, and were designated the

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sparse and dense stations, respectively. A 50-m line (marked every 5 m) was staked to the bottom at both sites. The lines were perpendicular to the general direction of sand ripple marks on the sea floor. The ends of the lines were marked with surface buoys and a large metal barrel or beam that gave a distinct trace on record. As a result, each 50-m line could be located on a record and placed within a known pattern of excavations.

The side-scan sonar was a 500 kHz system (Klien 521 dual channel side-scan). Recordings were made on wet paper at 60 lines/cm. All records were made on the 50-m range scale, giving a record with a 50-m width on both sides of the tow fish (a hydrodynamically designed body containing the underwater transducers). The record was uncorrected for ship speed and depth of tow (see below). The fish was towed at a depth of 5–6 m above the bottom (45–60° wire angle), at constant rpm, and at a constant compass direction. We used two boats with deep V-hulls: a 21-foot Lucas (Hurricane 600) and a 40-foot converted Bristol Bay fisheries boat (R/V ALTA). While the side-scan was towed under a variety of sea conditions, quantitative measurements were taken only from records made in seas with <0.5-m swell and no wind chop. Divers placed sea floor targets 50 meters apart within the study area. These were visible on the side-scan displays, allowing the records to be corrected for ship speed. This was done by digitizing the records and redrawing them to the correct scale using a computer.

Diver estimates of percent bottom covered by excavations, mean excavation size, and size distribution of excavations all came from diver maps of the dense and sparse station areas. Parallel estimates were made in several ways from side-scan records. Excavation patterns usually were measured only from a single run over a diver station. If estimates came from a single run, the run is numbered (run 1 or run 2). A composite sample was taken by examining all the single runs over a diver station and locating as many of the diver-observed excavations as possible. Finally, several single runs over the diver stations and over nearby areas were sampled to make regional estimates of excavation patterns. The regional areas were larger than the station area, but still represented the relatively sparse or dense feeding records.

All the underwater observations were done by divers using SCUBA. Divers located and examined all feeding excavations at least 20 m on both sides and at the ends of the 50-m lines. They measured to the nearest 0.5 m the relative position, shape, major dimensions, and depth (to nearest cm) and noted edge conditions (steep or gently sloping) for each excavation. These observations were facilitated by good water clarity (5–8 m). All excavations were less than one month old (Oliver *et al.*, 1984).

Results

The dense and sparse areas were easily distinguished by side scan. The percentage of the sea floor covered by feeding excavations was significantly greater at the dense compared to the sparse station ($P < 0.05$, Mann Whitney U-test). The mean size of excavations was significantly larger ($P < 0.05$, Mann Whitney U-test) at the dense station (Table II). There was also a significantly greater proportion of large excavations at the dense station ($P < 0.05$, Kolmogorov-Smirnov test). In all estimates from the same station, the three samples from side-scan records (single runs, composites, regional samples) were not significantly different from each other ($P > 0.05$, same tests). Finally, the dense study site was not located in the most intensely disturbed feeding area, where we found over 30% of the sea floor covered with feeding excavations.

Quantitative measurements of the feeding record made by divers were similar to quantitative estimates from side-scan records. Diver and side-scan measures of the

TABLE I

Percent area of the sea floor covered with gray whale feeding excavations at the relatively sparse and dense feeding areas in Pachena Bay

	Diving observations	Side Scan*		
		Run 1	Run 2	Composite
Dense feeding station ($6450\ m^2$)	11.7%	9.3%	9.8%	10.8%
Number of excavations	42	31	34	40
Sparse feeding station ($2680\ m^2$)	4.5%	3.4%	4.9%	3.6%
Number of excavations	14	8	11	12

* See Methods section for explanation of single run *versus* composite.
Diver and side-scan estimates are similar.

percentage of bottom covered by feeding excavations were remarkably similar (Table I). There was no significant difference ($P > 0.05$, Wilcoxon's signed-ranks test) between the mean size of excavations estimated by divers and by side scan at either the dense or sparse stations (Table II). At the dense station there was no significant difference ($P > 0.05$, Kolmogorov-Smirnov test) between diver and side-scan estimates of the size distribution of excavations (Fig. 1). However, at the sparse station, there was a significant difference ($P < 0.05$, Kolmogorov-Smirnov test) between diver and side-scan estimates of the size distribution of excavations (Fig. 2). This difference could only be detected when the side-scan records were examined from the entire region around the sparse station. This region was qualitatively similar to the station site, but contained more features. Since our sample size was small at the sparse station (Tables I, II), the regional sample increased the power of the statistical test (Sokal and Rohlf, 1981). Furthermore, this difference could only be established by dividing the excavation area into 1 m^2 size classes rather than 5 m^2 (Fig. 2).

Smaller excavations were more difficult to detect on side-scan records (Table III). At the dense station, only two excavations were never located on the records, both were $<3\ m^2$. Eighteen excavations between 3 and 13 m^2 were missed on at least one record at the dense station. No excavations larger than 13 m^2 were missed on records from either station (Table III).

TABLE II

Mean size (m^2) of excavations estimated by divers and side scan in the relatively dense and sparse feeding areas in Pachena Bay (± standard errors)

	Diving observations	Side scan*		
		Run 1	Run 2	Composite
Dense feeding station ($6450\ m^2$)	18.0 ± 2.6	18.1 ± 3.4	18.6 ± 2.6	17.4 ± 2.4
Number of excavations	42	31	34	40
Sparse feeding station ($2680\ m^2$)	8.6 ± 2.8	11.5 ± 3.2	12.0 ± 3.3	8.0 ± 3.2
Number of excavations	14	8	11	12

* See Methods section for explanation of single run *versus* composite.

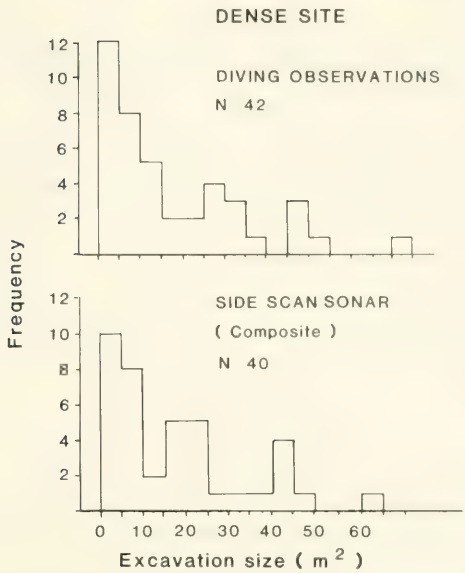

FIGURE 1. Size distribution of feeding excavations measured by divers and from side-scan records (a composite sample) at the dense station.

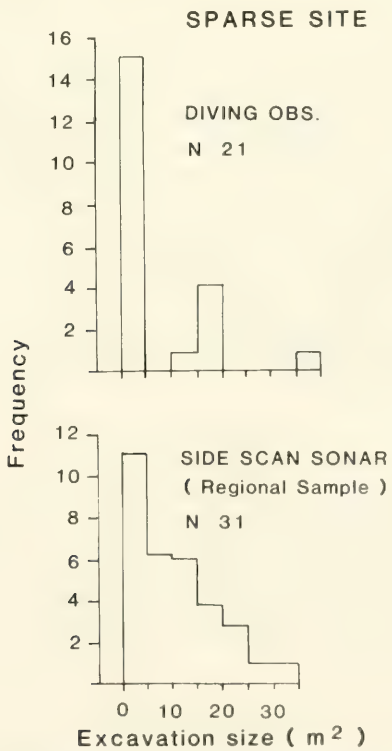

FIGURE 2. Size distribution of feeding excavations measured by divers and from side-scan records (regional sample) at the sparse station.

TABLE III

The ability of side scan to detect relatively small (<13 m²) or large excavations (>13 m²)

	<13 m²		>13 m²	
	Never seen	Missed at least once	Never seen	Missed at least once
Dense feeding area	9%	43%	0%	0%
Number of excavations	23		19	
Sparse feeding area	27%	78%	0%	0%
Number of excavations	9		5	

Only a few excavations were never located by side scan; more were missed on at least one run over the station areas.

DISCUSSION

Side-scan sonar can give an excellent impression of the relative intensity of gray whale feeding in soft-bottom habitats. Some feeding excavations are undetected because of their small size or orientation to the sonar signal. Nevertheless, side-scan and diver estimates of excavation sizes and the percent area disturbed show the same relative differences between a dense and a sparse feeding record, even when the dense and sparse records were relatively similar. The feeding record is quantified more precisely and easily, and for large areas more effectively, from records than from diver observations.

Side-scan sonar has considerable potential as a tool for documenting feeding patterns of gray whales. Spatial and temporal variations in the relative intensity of feeding can be documented. Ideas about large-scale patterns of habitat and prey utilization can be tested. Side scan also has applications in future management of bottom-feeding marine mammals such as the gray whale, and perhaps the walrus (Oliver *et al.*, 1983a). Just as the browse patterns of deer and other ungulates are used by terrestrial biologists (de Voos and Mosby, 1971), excavation patterns documented by side scan can help to assess the interactions between large marine grazers and their benthic food. The most important contribution of side-scan sonar to future management may be in evaluating long-term ecological questions involving the exploitation of peripheral and central feeding grounds as the gray whale population grows, stabilizes, or declines in size.

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LITERATURE CITED

BOGOSLOVSKAYA, L. S., L. M. VOTROGOV, AND T. N. SEMENOVA. 1981. Feeding habits of the gray whale off Chukotka. *Rep. Int. Whaling Comm.* **31**: 507–510.

JOHNSON, K. J., C. H. NELSON, AND H. L. MITCHELL. 1983. Assessment of gray whale feeding grounds and sea floor interaction. *USGS Open-File Report* 87–727.

NELSON, C. H., D. R. THOR, M. W. SANDSTROM, AND K. A. KVENVOLDEN. 1979. Modern biogenic gas-generated craters (sea-floor "pockmarks") on the Bering Shelf, Alaska. *Geol. Soc. Am. Bull.* **90:** 1144–1152.

OLIVER, J. S., P. N. SLATTERY, E. F. O'CONNOR, AND L. F. LOWRY. 1983a. Walrus, *Odobenus rosmarus*, feeding in the Bering sea: a benthic perspective. *Fish. Bull.* **81:** 501–512.

OLIVER, J. S., P. N. SLATTERY, M. A. SILBERSTEIN, AND E. F. O'CONNOR. 1983b. A comparison of gray whale, *Eschrichtius robustus*, feeding in the Bering Sea and Baja California. *Fish. Bull.* **81:** 513–522.

OLIVER, J. S., P. N. SLATTERY, M. A. SILBERSTEIN, AND E. F. O'CONNOR. 1984. Gray whale feeding on dense ampeliscid amphipod communities near Bamfield, British Columbia. *Can. J. Zool.* **62**(1): 41–49.

RAY, G. C., AND W. E. SCHEVILL. 1974. Feeding of a captive gray whale, *Eschrichtius robustus*. *Mar. Fish. Rev.* **36:** 31–38.

REIMNITZ, E., P. W. BARNES, L. J. TOIMIL, AND J. MELCHIOR. 1977. Ice gouge recurrence and rates of sediment reworking, Beaufort Sea, Alaska. *Geology* **5:** 405–408.

RICE, D. W., AND A. A. WOLMAN. 1971. The life history and ecology of the gray whale (*Eschrichtius robustus*). *Am. Soc. Mammal. Spec. Publ.* **3:** 1–142.

SOKAL, R. R., AND F. J. ROHLF. 1981. *Biometry*. W. H. Freeman and Company, San Francisco. 859 pp.

THOR, D. R., AND C. H. NELSON. 1981. Ice gouging on the subarctic Bering shelf. Pp. 279–292 in *The Eastern Bering Sea Shelf: Oceanography and Resources, Vol. 1*, D. W. Hood and J. A. Calder, eds. Vol. 1, Univ. of Washington Press, Seattle.

DE VOOS, A., AND H. S. MOSBY. 1971. Habitat analysis and evaluation. Pp. 135–172 in *Wildlife Management Techniques* (3rd ed.), R. H. Giles, ed. The Wildlife Society.

ZIMUSHKO, V. V., AND M. V. IVASHIN. 1980. Some results of Soviet investigations and whaling of gray whales (*Eschrichtius robustus* Lilljeborg, 1961 [sic, for 1861]). *Rep. Int. Whaling Comm.* **30:** 237–246.

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ERRATUM

THE BIOLOGICAL BULLETIN, Volume 166, Number 3, Page 529

The following correction should be made in the paper by Chizuko Obata and Shin-Ichi Nemoto entitled, Artificial parthenogenesis in starfish eggs: production of parthenogenetic development through suppression of polar body formation by methlxanthines (1984, *Biol. Bull.* **166:** 525–536): the scale bar in A of Figure 4 should read 150 μm. Thus the last sentence of the legend should read:

Bar in A representing 150 μm is common to A through F.

Reference: *Biol. Bull.* **167:** 271–309. (October, 1984)

THE PREVENTION OF POLYSPERMIC FERTILIZATION IN SEA URCHINS[1]

HERBERT SCHUEL

Department of Anatomical Sciences, University at Buffalo, SUNY, Buffalo, New York 14214 and Marine Biological Laboratory, Woods Hole, Massachusetts 02543

INTRODUCTION

During fertilization a single haploid sperm nucleus fuses with the haploid egg nucleus to establish the normal diploid chromosomal complement of the new individual. This is a critical event in the initiation of development because polyspermy, the fusion of more than one sperm pronucleus with the female pronucleus, results in abnormal development and the eventual death of the embryo (Lillie, 1919; Rothschild, 1956).

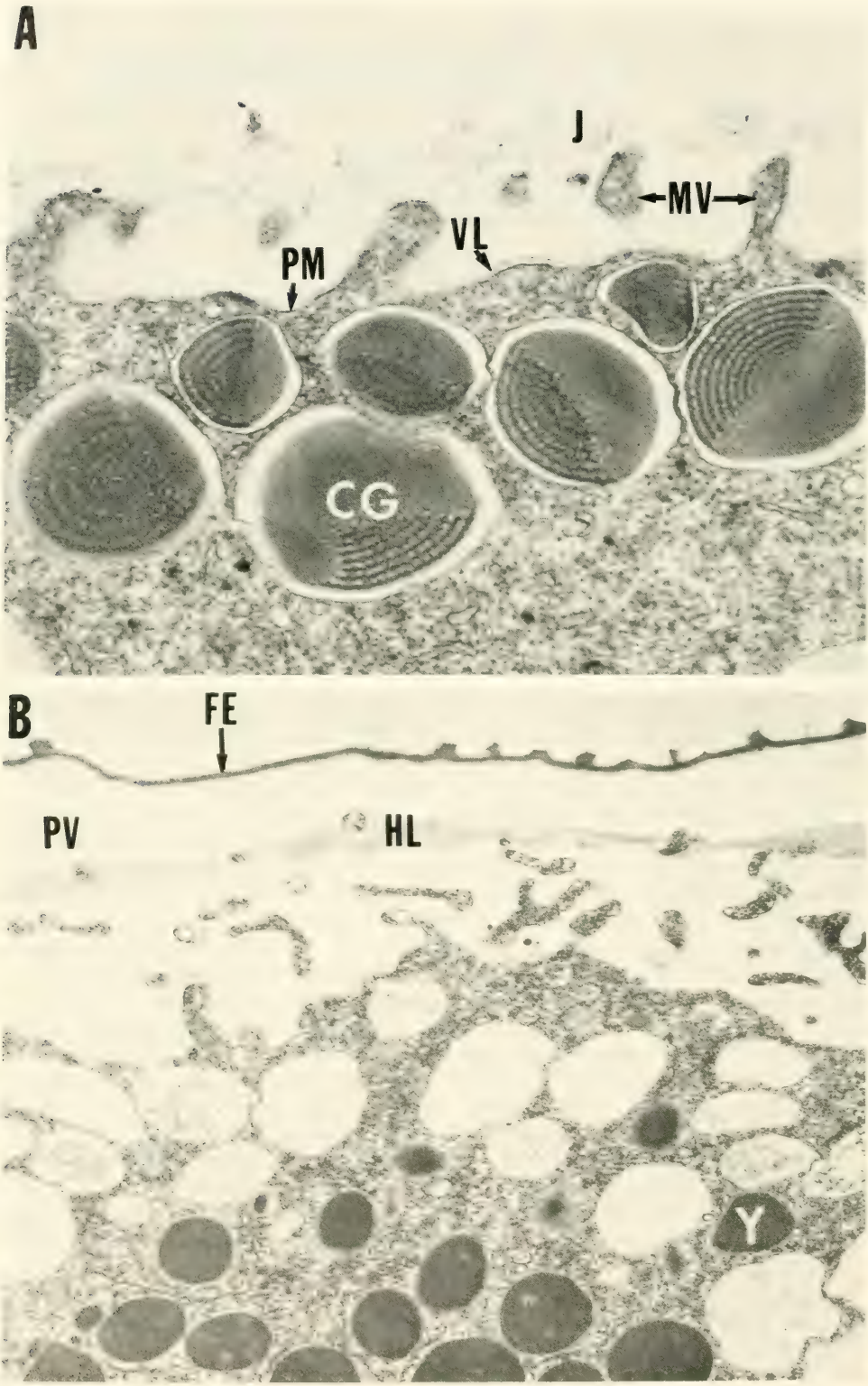
Various strategies have evolved to assure monospermic fertilization. Several sperm normally may enter large yolky eggs (certain insects, mollusks, sharks, reptiles, birds, urodels, etc.) but only one fuses with the egg's pronucleus (Lillie, 1919; Rothschild, 1956). Supernumerary sperm nuclei within the cytoplasm of these eggs undergo degeneration. The mechanism responsible for this process is poorly understood (Jaffe and Gould, 1984). In sea urchins and most other animals including mammals polyspermy is prevented by blocks that operate at the egg surface to prevent supernumerary sperm from entering the egg's cytoplasm (reviewed by Lillie, 1919; Allen, 1957; Rothschild, 1956; Runnstrom, *et al.,* 1959; Runnstrom, 1966; Ginzburg, 1972; Gwatkin, 1977; Epel, 1978; Schuel, 1978, 1984; Dale and Monroy, 1981; Wolf, 1981; Whitaker and Steinhardt, 1982; Schmell *et al.,* 1983; Jaffe and Gould, 1984; Nuccitelli and Grey, 1984). The purpose of this review is to examine all processes that are currently known to contribute to the prevention of polyspermy in sea urchins.

Sea urchin gametes are an ideal model system to study polyspermy preventing mechanisms as well as other aspects of fertilization and development. Fertilization in echinoderms normally takes place externally in sea water which facilitates culturing and experimental manipulation of gametes in the laboratory. The sexes are separate and large quantities of gametes can be collected from adult animals (Harvey, 1956). For example, a single female *Arbacia* yields 5 ml of eggs containing approximately 4×10^6 ova, while a male of this species yields 5 ml of semen containing about 10^{11} sperm. The eggs undergo synchronous development following fertilization under laboratory conditions. These features make it possible to study polyspermy-preventing mechanisms by biochemical, physiological, and morphological techniques that are difficult to apply to other types of eggs. This is especially true with respect to mammalian eggs which share many functional aspects of fertilization in common with sea urchins (Gwatkin, 1977; Schuel, 1978; Lopo, 1983; Schmell *et al.,* 1983).

Polyspermy can be quantitated by counting the number of sperm pronuclei within the cytoplasm of sea urchin eggs fixed prior to the time of pronuclear fusion (Presley and Baker, 1970; Byrd and Collins, 1975; Schuel and Schuel, 1981), or by counting the incidence of multipolar divisions at the time of first cleavage (Wilson, 1900; Clark,

Received 16 June; accepted 24 July 1984.

[1] Dedicated to the late Professor E. E. Just, in commemoration of the hundredth anniversary of his birth.


A
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1936; Schuel *et al.*, 1973). Monospermic eggs divide into two blastomeres at first cleavage, while polyspermic eggs either divide into more than two cells or can be seen to contain multiple asters.

The Egg Surface and Investing Coats

Since polyspermy in sea urchins is prevented by processes that operate at the egg surface, the morphological and biochemical properties of the egg surface and investing coats must be considered, as well as changes that take place in these structures during fertilization. These surface structures include the jelly coat, vitelline layer, plasma membrane (oolemma), and subjacent cortical granules (Fig. 1).

The mature sea urchin egg is surrounded by a jelly coat that hydrates and expands upon contact with sea water. The jelly slowly dissolves, charging the surrounding sea water with its biochemical components. The jelly coat can be removed from the eggs by treating them briefly with acidified (pH 5.0) sea water, by straining through bolting cloth, and by other treatments (Harvey, 1956). The jelly coat is transparent because its refractive index is the same as sea water, but its presence is indicated by the spacing between individual eggs. If the eggs are contiguous, there is no jelly coat. However, some jelly coat material remains attached to the surfaces of acid-dejellied eggs under such circumstances (Vacquier *et al.*, 1979). The jelly coat is known to contain glyco (sialo)-proteins, fucose sulfate polysaccharides, and small peptides (reviewed by: Runnstrom *et al.*, 1959; Tyler and Tyler, 1966a; Metz, 1967; Lopo, 1983).

Sperm must swim through the jelly coat and its dissolved components to reach the surface of the egg. As they do, they are activated by molecular constituents of the jelly as evidenced by increased motility, increased respiration, a transient isoagglutination, and induction of the acrosome reaction (Lillie, 1919; Tyler and Tyler, 1966a, b; Metz, 1967; Summers *et al.*, 1975; SeGall and Lennarz, 1979; Lopo, 1983; Monroy and Rosati, 1983). Increased sperm motility near eggs is an obvious advantage in increasing the probability of fertilization. The transient isoagglutination may perform a similar role by keeping sperm in the vicinity of eggs, especially in the animals' native marine habitat. The acrosomal filament that is formed at the apical end of the sperm by the acrosome reaction binds the sperm to the egg surface (vitelline layer) and fuses with the egg's plasma membrane to initiate sperm penetration (Dan, 1967; Colwin and Colwin, 1967; Summers *et al.*, 1975; Lopo, 1983). Acrosomal enzymes such as phospholipase A_2 (Conway and Metz, 1976), proteases (Levine and Walsh, 1979; Yamada and Aketa, 1981; Green and Summers, 1982), and arylsulfatase (Hoshi and Moriya, 1980) have been implicated in facilitating sperm penetration.

The vitelline layer is a thin extracellular coat attached to the external surface of the egg's plasma membrane (Fig. 1a). The vitelline layer is composed of glycoproteins and contains species specific sperm receptors (Summers *et al.*, 1975; Glabe and Vacquier, 1977; Schmell *et al.*, 1977; Glabe and Lennarz, 1981; Kinsey and Lennarz, 1981; Rossignol *et al.*, 1981; Lopo, 1983; Niman *et al.*, 1984). Monoclonal antibodies against vitelline layer proteins prevent sperm binding and fertilization (Gache *et al.*,

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FIGURE 1. Transmission electron micrographs showing surface morphology of sea urchin, *Strongylocentrotus droebachensis*, egg. Hylander (1984, unpubl. data). *A:* Unfertilized egg. The vitelline layer (VL) is attached to the outer surface of the egg's plasma membrane (PM). Numerous short microvilli (MV) are located at the egg surface. Cortical granules (CG) showing the spiral lamellae structure characteristic of this genus are located immediately subjacent to the plasma membrane. Jelly coat (J). 22,500×. *B:* Surface of fertilized egg fixed 60 min after insemination. The cortical granules have secreted their contents. The fertilization envelope (FE) is separated from the egg surface by the perivitelline space (PV). The hyaline layer (HL) invests the egg surface. Yolk platelet (Y). 20,300×.

1983). Cross species fertilization is facilitated by removal of the vitelline layer (Longo, 1977). The egg's plasma membrane (oolemma) exhibits many short microvilli. As is true for all living cells, the sea urchin egg maintains an unequal distribution of ions across its plasma membrane which is reflected in a resting potential of −60 to −70 mV. The fertilizing sperm triggers a rapid electrical depolarization of the oolemma which acts as a rapid block to polyspermy (reviewed by: Hagiwara and Jaffe, 1979; Whitaker and Steinhardt, 1982; Shen, 1983; Gould-Somero and Jaffe, 1984; Nuccitelli and Grey, 1984; also see *Electrical block* section, below).

Cortical granules, Golgi-derived secretory organelles, are located immediately subjacent to the plasma membrane (Fig. 1a). The morphology and biochemical composition of sea urchin cortical granules have been studied intensively (reviewed by: Runnstrom, 1966; Epel, 1978; Schuel, 1978, 1984; Shapiro and Eddy, 1980). The cortical granules form an irregular monolayer under the plasma membrane, with areas of tight packing interspersed with numerous patches of oolemma that are devoid of subjacent cortical granules. This packing arrangement is directly related to the sites where sperm penetration can take place and to the prevention of polyspermy (Schuel, 1978 and 1984, also see *Cortical granule protease* section, below).

The surface of the egg is drastically altered as a result of cortical granule secretion during fertilization (Fig. 1b). Cortical granule exocytosis begins at the site where the fertilizing sperm fuses with the oolemma, rapidly propagates around the entire surface of the sea urchin egg, and is completed within 1–2 min depending upon species and temperature (reviewed by: Schuel, 1978, 1984). Secretion of the cortical granules is triggered by an increased concentration of free calcium ions within the egg's cytoplasm (reviewed by: Epel, 1978; Schuel, 1978, 1984; Jaffe, 1980). A similar calcium transient triggers exocytosis in stimulated somatic secretory cells (Poste and Allison, 1973; Rubin, 1982). Released cortical granule contents promote the detachment of the vitelline layer from the egg's plasma membrane and its elevation from the egg surface to become the fertilization envelope (reviewed by: Epel, 1978; Schuel, 1978, 1984; Shapiro and Eddy, 1980). This process is known as the cortical reaction. The fertilization envelope (membrane) acts as a mechanical barrier to penetration by supernumerary sperm (see *Fertilization envelope* section, below). The hyaline layer is formed by secreted cortical granule product(s) as well. Its primary function is to act as an extracellular cement to maintain blastomere adhesion during cleavage (reviewed by: Schuel, 1978, 1984), but it can also assist in preventing polyspermy under appropriate conditions (see *Hyaline layer* section, below).

Cortical granule secretion is an important aspect of polyspermy preventing mechanisms in sea urchins. Immature oocytes at the germinal vesicle stage of development do not have cortical granules in association with their plasma membrane and therefore can not undergo a cortical reaction in response to stimulation by sperm. These immature oocytes are extremely vulnerable to polyspermy (Harvey, 1956; Longo, 1978; De Felice and Dale, 1979). Furthermore, treatments that inhibit cortical granule discharge promote polyspermy. These include: application of hydrostatic pressure (Chase, 1967); colchicine (Hagstrom, 1956a); narcotics such as urethane (Longo and Anderson, 1970b) and chloral hydrate (Hagstrom, 1956a; Lonning, 1967); nicotine (Hagstrom and Allen, 1956); enzymatic inhibitors of the cortical granule protease (Hagstrom, 1956a; Lonning, 1967; Longo and Schuel, 1973; Schuel *et al.*, 1973; Longo *et al.*, 1974; Alliegro and Schuel, 1984); and the phospholipase A_2 inhibitor quinacrine (Ferguson and Shen, 1984). Finally, premature discharge of the cortical granules induced by activating eggs with saponin or calcium ionophore A23187 prevents the penetration of subsequently added sperm (Ginzburg, 1964; Schuel *et al.*, 1976b). Sperm can penetrate eggs after parthenogenetic activation by ammonia, a

treatment that does not trigger premature secretion of the cortical granules (Schatten, 1978; Longo, 1983).

Reproduction in Nature

The properties of fertilization studied with sea urchin gametes in the laboratory reflects reproductive processes of the animals in their natural habitat. Prior to spawning, adult urchins migrate together to form very dense local populations. All the members of the local population spawn at the same time (Harvey, 1956; Boolootian, 1966; Reese, 1966). Other marine invertebrates exhibit similar spawning behavior (Harrison *et al.,* 1984). The synchronous release of large quantities of gametes is clearly advantageous for animals that reproduce externally. Since sperm must swim to the egg to fertilize it, the production of an excess number of sperm (approximately 10^4 to 10^5/egg) also favors fertilization (Harvey, 1956). The probability that an individual egg will be fertilized depends upon the number of sperm in its immediate vicinity, which in turn depends upon the proximity of an adult male to the spawning female and the direction of current flow. Given this set of circumstances, there must have been strong evolutionary pressures to select for fertilization mechanisms likely to be successful over a wide range of sperm/egg ratios. Thus, to increase the chances of a successful hit under conditions of a low sperm density, the unfertilized egg should be highly receptive to sperm with numerous functional receptors at its surface. To prevent polyspermy under conditions of a very high sperm density, the fertilized (activated) egg must be able to effectively exclude supernumerary sperm on its surface. These expected features have been documented in laboratory studies of sea urchin fertilization (see below).

Concepts of Fast and Permanent Polyspermy Blocks

Our conceptual framework for analysis of polyspermy prevention in sea urchins is based upon the responses of eggs to insemination with excess sperm (Fig. 2). Large numbers of sperm rapidly attach to the egg surface within seconds after insemination (Fig. 2a). The fertilization envelope lifts off the surface of the egg starting at the site of activation by the fertilizing sperm (Moser, 1939; Anderson, 1968; Green and Summers, 1980), spreads around the surface of the egg, and is completely elevated within about a minute (Fig. 2b–2d). Most of the sperm initially attached to the egg surface are detached as the fertilization envelope elevates. The fertilization envelope formed as a result of the cortical reaction functions as an absolute mechanical barrier to penetration by supernumerary sperm (Rothschild, 1956; Schuel, 1978, 1984). Even under the kind of conditions illustrated in Figure 2, polyspermy is a rare event since 85 to 95% of eggs in such cultures will admit only a single sperm (Ginzburg, 1964; Schuel and Schuel, 1981; Nuccitelli and Grey, 1984; Schuel *et al.,* 1984). Early students of fertilization had made similar observations and concluded that the fertilization envelope (permanent polyspermy block) did not elevate rapidly enough from the entire surface of the egg to be exclusively responsible for the prevention of polyspermy in the presence of excess sperm (Just, 1919; Lillie, 1919; Just, 1939). Hence, the belief arose that a more rapid block to polyspermy also must function to protect the egg until the permanent block is established by completion of the cortical reaction.

The existence of the putative rapid (pre-cortical) block to polyspermy was demonstrated by measuring the rates of initial fertilization and re-fertilization (polyspermy) in egg cultures upon insemination with excess sperm (Rothschild and Swann, 1952;

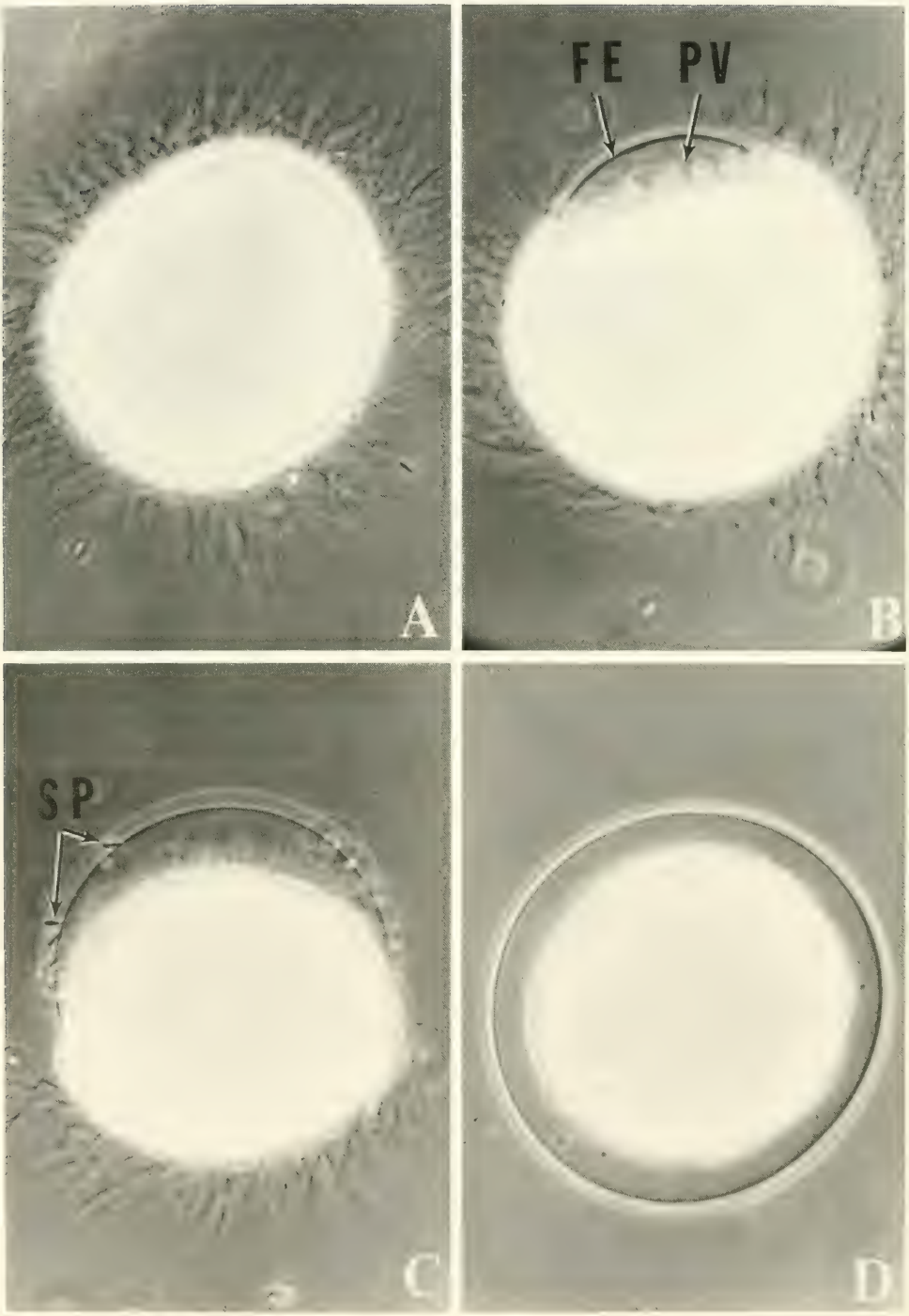

FIGURE 2. Fertilization reaction of *Lytechinus pictus* eggs upon insemination with excess sperm. From Vacquier and Payne (1973), reprinted with permission of Academic Press, Inc. *A:* Egg fixed at 10 s after insemination showing numerous sperm bound to the entire surface of the egg. *B:* Egg fixed at 25 s

Rothschild, 1956; Presley and Baker, 1979). In these studies the sperm were either removed by dilution or killed by addition of spermicides at various times after insemination. The percent of eggs in the cultures that were unfertilized, fertilized, monospermic, and polyspermic was determined. The first successful sperm-egg reaction occurs within seconds after insemination, while re-fertilization (polyspermy) occurs at a much slower rate and stops entirely at about 60 s when the cortical reaction has been completed (Rothschild and Swann, 1952). The receptivity of the fertilized egg to refertilization is reduced about 20 fold during this interval. The conduction time for the establishment of this rapid but incomplete block to polyspermy was estimated to be less than 1 s. Subsequent studies by Presley and Baker (1970) confirmed that the observed incidence of monospermic fertilization under such conditions is far greater than predicted if receptivity of the egg were not reduced after the first sperm-egg fusion. Together these findings show that some mechanism must operate prior to completion of the cortical reaction in sea urchins to reduce the probability of polyspermy (reviewed by: Whitaker and Steinhardt, 1984; Nuccitelli and Grey, 1984).

Nevertheless the existence of a rapid pre-cortical block to polyspermy has been questioned (Hagstrom and Allen, 1956; Kille, 1959; Byrd and Collins, 1975; Epel, 1978; DeFelice and Dale, 1979; Dale and Monroy, 1981; Dale *et al.*, 1982). The validity of this point of view must be examined in light of currently known properties of fertilization in sea urchins, and a consideration of how quickly a rapid block to polyspermy would have to be established in order to be effective.

The half time for eggs to be fertilized upon insemination with excess sperm is about 0.5 to 1.0 s (Rothschild and Swann, 1952; Byrd and Collins, 1975; Schuel and Schuel, 1981). Since a portion of this period must represent the time required for the sperm to reach to the egg surface, the reaction of the fertilizing sperm with its receptor must be extremely rapid. Sperm binding to receptors in the vitelline layer is a prerequisite for fertilization (Summers *et al.*, 1975; Glabe and Vacquier, 1978). There are 1500 to 6000 sperm binding sites on the surface, presumably the vitelline layer of the unfertilized sea urchin egg (Vacquier and Payne, 1973; Decker and Lennarz, 1979; Green, 1983). How many of these correspond to potential penetration sites is unknown. However, the penetration sites must be numerous since eggs can be fertilized upon insemination with as few as 10 sperm/egg (Byrd and Collins, 1975; Schmell *et al.*, 1977). Another important factor is the interval between activation of the egg by the fertilizing sperm and completion of the cortical reaction. Cortical granule exocytosis begins within 3–40 s of activation and is completed within 1–2 min (reviewed by: Schuel, 1978, 1984; Nuccitelli and Grey, 1984). The range of values reported in the literature probably reflects species differences, temperature differences, and factors associated with the experimental criteria used to quantitatively evaluate exocytosis. In any case these observations show that a significant portion of the egg's surface remains unprotected by an absolute mechanical block to polyspermy (fertilization envelope) for an appreciable time until the cortical reaction is completed. Under these circumstances polyspermy would be the rule rather than the exception even at low or moderate sperm densities in the absence of a rapid block. To be effective the rapid block would have to be established within a fraction of a second

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after insemination showing early stage in elevation of the fertilization envelope (FE) and development of the perivitelline space (PV). Sperm detachment from the elevating fertilization envelope is evident. *C:* Egg fixed at 35 s. The cortical reaction has spread around more of the egg surface. Note that a few sperm (SP) remain bound to the partially elevated fertilization envelope. *D:* Egg fixed at 50 s. The fertilization envelope is elevated over the entire surface of the egg and detachment of bound sperm has been completed. 400×.

after the first successful sperm-egg reaction to prevent supernumerary sperm from entering (see *Electrical block* section, below).

Thus, the rapid block to polyspermy should no longer be regarded as a controversial issue. Rather it should be recognized as a physiological necessity for normal fertilization in sea urchins, and in many other organisms as well. Our attention now should be directed toward identifying the processes that act to limit sperm penetration prior to completion of the cortical reaction and to the elucidation of their mechanisms of operation.

Multiple Polyspermy Blocks

Although it is conceptually convenient to think of polyspermy prevention in terms of separate rapid and permanent blocks, it is now evident that several processes act together to reduce the probability of polyspermic fertilization in sea urchins. The role of the elevated fertilization envelope as a mechanical barrier to polyspermy has been recognized for over a century (Fol, 1877; Hertwig and Hertwig, 1887). Other processes involving ion-dependent changes in membrane potential (Jaffe, 1976, 1980; Schuel and Schuel, 1981), various secretory products released by the egg's cortical granules (reviewed by: Epel, 1978; Schuel, 1978, 1984), release of peroxide to inactivate sperm (Boldt *et al.,* 1981), production of arachidonic acid oxidation products (Moss *et al.,* 1983; Schuel *et al.,* 1984), and the protective (?) role of the jelly coat (Hagstrom, 1956b) have been discovered during the past 30 years. The list is probably still incomplete. These multiple defenses tend to limit sperm penetration by supernumerary sperm even when any one of the polyspermy blocking systems is inhibited experimentally. Even in the presence of an effective polyspermy promoting agent, the induction of polyspermy also depends upon the sperm density in the cultures since sufficient numbers of sperm must be present to overwhelm the egg's remaining defenses (Boldt *et al.,* 1981; Schuel and Schuel, 1981; Schuel *et al.,* 1973; Schuel *et al.,* 1984). The existence of these multiple defenses also can be inferred from the wide variety of agents that are known to cause polyspermy in sea urchins (Hertwig and Hertwig, 1887; Clark, 1936; Harvey, 1956; Schuel, 1978, 1984; Dunham *et al.,* 1982; Alliegro and Schuel, 1984).

Jelly coat

The jelly coat that invests the surface of the sea urchin egg may act as a filter to regulate the arrival of sperm at the egg surface (vitelline layer) and thereby reduce the probability of polyspermic fertilization. Evidence for such a role is provided by observations that eggs fertilize more rapidly and are more vulnerable to polyspermy after the jelly coat is removed (Hagstrom, 1956b, 1959; Runnstrom *et al.,* 1959; Schuel *et al.,* 1974; Vacquier *et al.,* 1979; Schuel and Schuel, 1981). However, there is considerable variability in the reported susceptibility of eggs to polyspermy after removal of the jelly coat, with some studies showing a large increase (Hagstrom, 1956b) and others a much smaller but still detectable increase (Schuel *et al.,* 1974; Schuel and Schuel, 1981). This may reflect species differences in the importance of the intact jelly coat in preventing polyspermy. Alternatively, it may not be the absence of the jelly coat *per se,* but rather damage produced by the treatment used to remove the jelly coat from the eggs that is responsible for the increased polyspermy (Hagstrom, 1959). This possibility also is suggested by abnormalities in the electrical properties of the egg's plasma membrane and the high levels of polyspermy sometimes seen in acid de-jellied eggs (DeFelice and Dale, 1979; also see *Electrical block* section, below). Furthermore, arylsulfatases isolated from sea urchin sperm or from limpets can dissolve

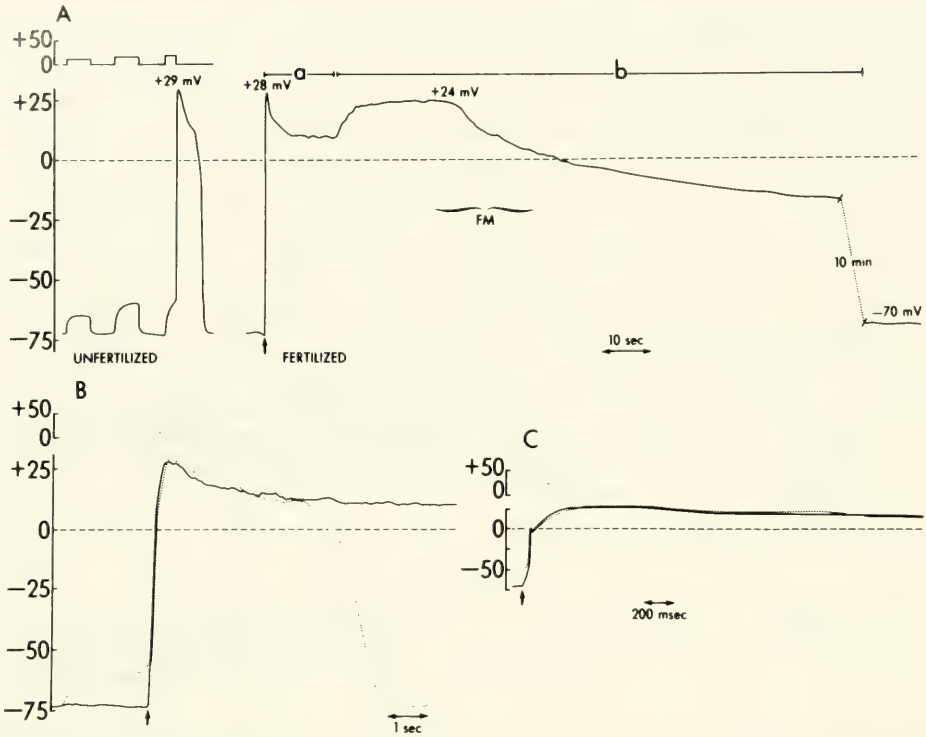
the jelly coat but do not cause polyspermy (Hoshi and Moriya, 1980), even when treated eggs are inseminated with excess sperm (Schuel and Schuel, unpubl. data). Additional work is required to resolve these questions.

The ambient sea water near the eggs contains dissolved jelly coat components which affect sperm function in a variety of ways (see The Egg Surface and Investing Coats section, above). Of these, the transient isoagglutination and induction of the acrosome reaction also may be contributing factors in preventing polyspermy. Since agglutinated sperm are not free to react with eggs until they disperse, this process may tend to reduce temporarily the frequency of sperm-egg collisions. The infertility of sperm that are permanently agglutinated by antibodies is consistent with this notion (Metz *et al.*, 1964). Induction of the acrosome reaction is followed by a rapid decline in the fertilizing capacity of sperm (Tyler and Tyler, 1966b; Kinsey *et al.*, 1979; Vacquier, 1979; Sano and Kanatani, 1980; Christen *et al.*, 1983; Nakano *et al.*, 1984). Such a reduction in the number of potent sperm near already fertilized eggs would be expected to have a salutary effect in reducing the chances of re-fertilization (polyspermy). Quantitative studies are required to determine whether these processes actually have a significant physiological role in preventing polyspermy.

Electrical block

Electrical depolarization of the plasma membrane is a universal response of cells including eggs to stimulation (Tyler *et al.*, 1956a). Jaffe (1976) discovered that sea urchin eggs use this process to establish a rapid but transient block to polyspermy (reviewed by: Hagiwara and Jaffe, 1979; Dale and Monroy, 1981; Whitaker and Steinhardt, 1982; Shen, 1983; Nuccitelli and Grey, 1984; Gould-Somero and Jaffe, 1984; Jaffe and Gould, 1984; Schuel, 1984). Jaffe's electrophysiological studies showed that: (1) the resting membrane potential of unfertilized eggs is −70 mV; (2) the oolemma depolarizes to +10 to +20 mV with 3 s of insemination; (3) the depolarized state is maintained for about 60 s until the cortical reaction is completed; (4) thereafter the oolemma repolarizes to about −70 mV during the next 5–10 min; and (5) eggs which fail to depolarize to above −10 mV become polyspermic, while those that depolarize to +10 to +20 mV are typically monospermic. Finally, fertilization is prevented if the membrane potential is set at +5 mV but occurs at −10 mV. The electrical block is not absolute since some of the unfertilized eggs voltage clamped at +10 to +20 mV do fertilize at high sperm densities while none of these clamped eggs fertilize at low to moderate sperm densities (Jaffe, 1976; Jaffe *et al.*, 1982; Lynn and Chambers, 1984). Nicotine can cause polyspermy by inhibiting the electrical block (Jaffe, 1980).

The kinetics and ionic basis for the fertilization (activation) potential in sea urchin eggs has been studied intensively (reviewed by: Hagiwara and Jaffe, 1979; Whitaker and Steinhardt, 1982; Shen, 1983; Nuccitelli and Grey, 1984). Its major features are illustrated (Figs. 3–6) from the work of Chambers and DeArmendi (1979). Normal unfertilized eggs show a resting potential of −70 to −80 mV (Fig. 3a). The membrane is electrically excitable and responds to stimulation with a positive going action potential of brief (3.5–14.6 s) duration. Sperm triggers a long lasting biphasic (segments *a* and *b*) fertilization potential which remains positive until fertilization envelope elevation is completed. The initial phase of segment *a* of the fertilization potential corresponds to an action potential that is triggered by the fertilizing sperm (Fig. 3b). Segment *b* begins at the end of segment *a* (about 15 s) and reaches a maximum at 33 s. Depolarization begins when the sperm reacts with its receptor at the egg surface (DeFelice and Dale, 1979; Hulser and Schatten, 1982), and attains levels (+5 to +20 mV)



FIGURES 3–6. Electrical recordings of fertilization potentials in *Lytechinus variegatus* eggs. *Abscissa:* time (see scales); *ordinate:* (*upper*) current in pA; (*lower*) membrane potential in mV. Vertical arrow: start of depolarization in inseminated egg; horizontal bracket: interval during which fertilization membrane (FM) elevation occurred. Temp. = 22°C. From Chambers and DeArmendi (1979), reprinted with permission of Academic Press, Inc.

FIGURE 3. Action potential and fertilization potential recorded in an egg cultured in sea water. *A:* Oscillograph record of unfertilized egg (*left*) injected with pulses of depolarizing current. An action potential was elicited by 20 pA pulse of 2 s duration. The pulse was cut off during the rising phase of the action potential. The same egg (*right*) after insemination. For segments (*a*) and (*b*), see text. Break in curve represents 10 min period when membrane potential repolarized steadily to −70 mV. *B:* Same oscillograph records as *A*, with time scale expanded 8×. The steep rising limb of the action potential (· · · · ·) and the fertilization potential (– – –) are superimposed. *C:* Same record as *B*, but time scale expanded 4× (32× scale of A).

capable of excluding sperm (Jaffe, 1976) within 100 m s (Fig. 3c). Repolarization of the fertilized egg is completed in 10 min, and is associated with increased potassium conductance (Steinhardt *et al.*, 1971). Damaged eggs have leaky membranes and show a resting potential of −10 to −20 mV (Fig. 4). They can not be excited electrically. Upon insemination, the sharp early peak of the fertilization potential (segment *a*) is missing. The membrane potential slowly rises to positive levels (segment *b*) within about 15 s. Such eggs lack a voltage sensitive mechanism to generate a rapid spike depolarization, which can be triggered only if the egg's membrane potential is more negative than −40 mV. Eggs with leaky membranes can be sealed and the resting potential restored to −70 mV by the transient injection of negative current (Chambers and DeArmendi, 1979). The rapid influx of calcium (Azarnia and Chambers, 1976; Paul and Johnston, 1978) and sodium (Payan *et al.*, 1981; Sardet *et al.*, 1982) ions

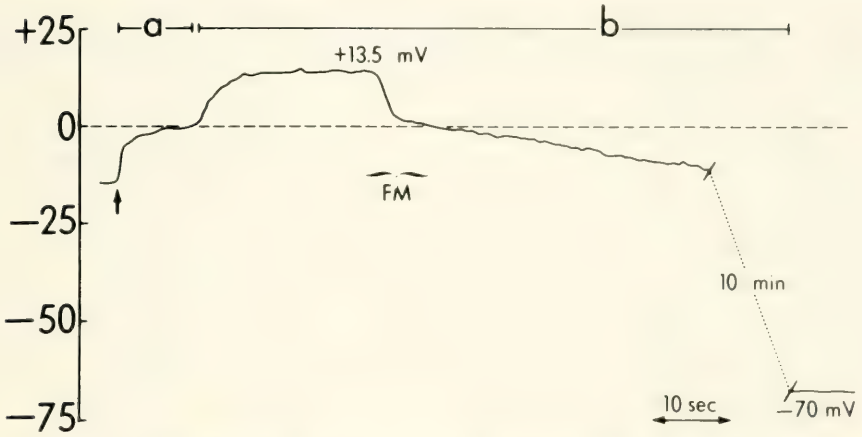


FIGURE 4. Fertilization potential of an egg in sea water whose resting potential was −15 mV.

at fertilization appears to be the ionic basis for the fertilization potential. This hypothesis is supported by recordings of fertilization potentials generated by eggs inseminated in sodium and calcium-depleted sea water. In low (4.8 m*M*) sodium (choline-substituted sea water), the membrane of the unfertilized egg is electrically excitable and can generate action potentials of briefer than normal duration upon stimulation (Fig. 5). After insemination these eggs fire a very brief action potential, and the fertilization potential (both segments *a* and *b*) is suppressed greatly. In low calcium sea water, the first component (segment *a*) of the fertilization potential is suppressed (Fig. 6). These findings show that both calcium and sodium influx generate the first response phase (segment *a*) of the fertilization potential, while sodium influx generates the second response phase (segment *b*).

These electrophysiological studies make it possible to predict key properties of the rapid electrical block to polyspermy:

(1) Unfertilized eggs that are not electrically excitable and/or that have low (−10 to −20 mV) resting potentials are abnormal or damaged, and should be susceptible

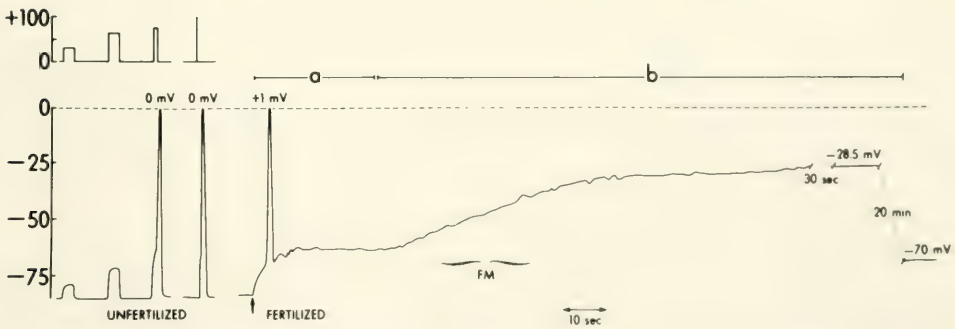


FIGURE 5. Action potential and fertilization potential recorded in an egg cultured in choline substituted sea water containing 4.8 m*M* Na^+. Action potentials of briefer than normal duration (compare with Fig. 3A, above) were elicited in the unfertilized egg (*left*) by pulses of 65 pA 1.2 s and 100 pA 100 ms duration. The same egg (*right*) after insemination.

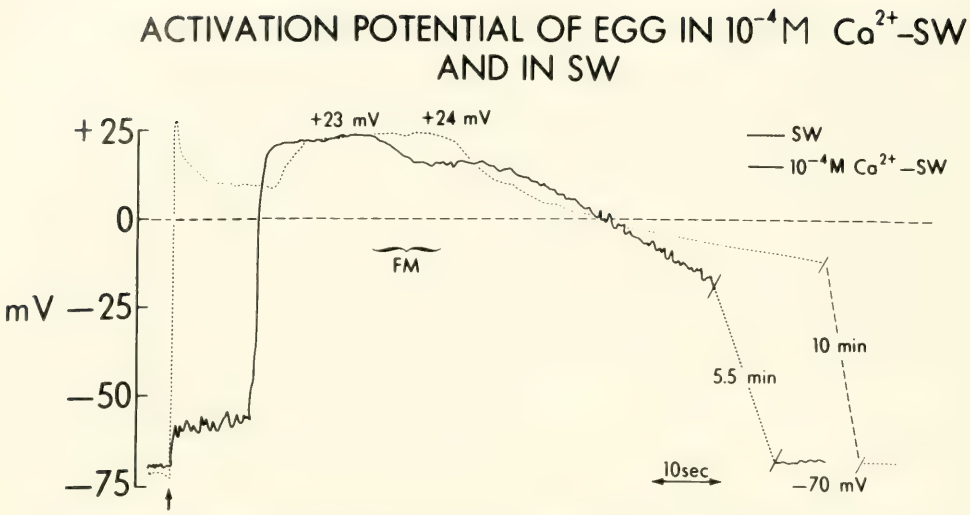


FIGURE 6. Recording of fertilization potential of egg inseminated in sea water containing 0.1 m*M* Ca^{++} (——), compared with egg fertilized in normal (10 m*M* Ca^{++}) sea water (· · · · ·).

to polyspermy. This expectation has been realized since such eggs are highly vulnerable to polyspermy (Jaffe, 1976; DeFelice and Dale, 1979; Whitaker and Steinhardt, 1983).

(2) Eggs fertilized in low sodium sea water, which suppresses the fertilization potential (Fig. 5), should be highly susceptible to polyspermy. This expectation has been realized since polyspermy results when eggs are fertilized in sodium depleted (choline-substituted) sea water (Nishioka and Cross, 1978; Jaffe, 1980; Schuel and Schuel, 1981). However, although a high incidence of polyspermy is obtained, the eggs do not fill up with supernumerary sperm when heavily inseminated in low sodium sea water (Schuel and Schuel, 1981). Two factors tend to limit sperm penetration under these conditions: the normal completion of the cortical reaction (Schuel and Schuel, 1981), and the adverse effects of the low sodium environment on sperm fertility (Schuel and Schuel, 1981; Bibring *et al.*, 1984).

(3) Eggs fertilized in calcium-depleted sea water, which suppresses the first component (segment *a*) of the fertilization potential (Fig. 6), should be susceptible to polyspermy. Such a phenomenon might account, in part, for the elevated polyspermy seen in 80% isotonic magnesium chloride/20% sea water (Clark, 1936). Magnesium is a potent ionic antagonist of calcium (Heilbrunn, 1956), and could suppress the calcium component of the fertilization potential (Jaffe, 1980). Increased polyspermy was not observed when eggs were fertilized with acrosome reacted sperm in zero calcium sea water (Schmidt *et al.*, 1982). However, sperm fertility is reduced 20× in zero calcium sea water, and these authors may not have used sufficient numbers of sperm to challenge the egg's remaining defenses.

The experiments with low sodium and zero calcium sea water illustrate an important point. The results of polyspermy inducing treatments can be complicated if the treatment also injures the sperm and thereby reduces its fertilizing capacity. Normal sperm function can not be assumed. It must be evaluated.

The cortical reaction normally propagates around the egg surface while the rapid electrical block is believed to be operational. In order to measure the duration and efficacy of the rapid block in *Arbacia,* advantage was taken of the fact that the cortical

granule secretion mediated block can be suppressed by soybean trypsin inhibitor (Schuel and Schuel, 1981). The rationale for these experiments was based on previous observations that a soybean trypsin inhibitor (SBTI) sensitive protease is secreted by the egg's cortical granules at fertilization (Schuel *et al.*, 1973; Vacquier *et al.*, 1973), that SBTI inhibits the cortical reaction and promotes polyspermy (Hagstrom, 1956a; Lonning, 1967; Longo and Schuel, 1973), that SBTI-treated eggs are monospermic initially and gradually become polyspermic with time (Hagstrom, 1956a; Longo and Schuel, 1973; Longo *et al.*, 1974; Schuel *et al.*, 1976a), and that all potential sperm penetration sites remain available at the surface of SBTI-treated *Arbacia* eggs for 3 min post insemination (Schuel *et al.*, 1976a; also see *Cortical granule protease* section, below). Under these conditions the time at which the SBTI-treated eggs first became polyspermic (Schuel and Schuel, 1981) was used to titrate the decay of the rapid block (Fig. 7). The half time for the first supernumerary sperm fusion event in natural (425 m*M* Na^+) sea water is 89.9 ± 4.7 s, compared to 15.8 ± 1.6 s in 26 m*M* Na^+ ($P < 0.01$). Furthermore, the decay in the rapid block corresponded to the period

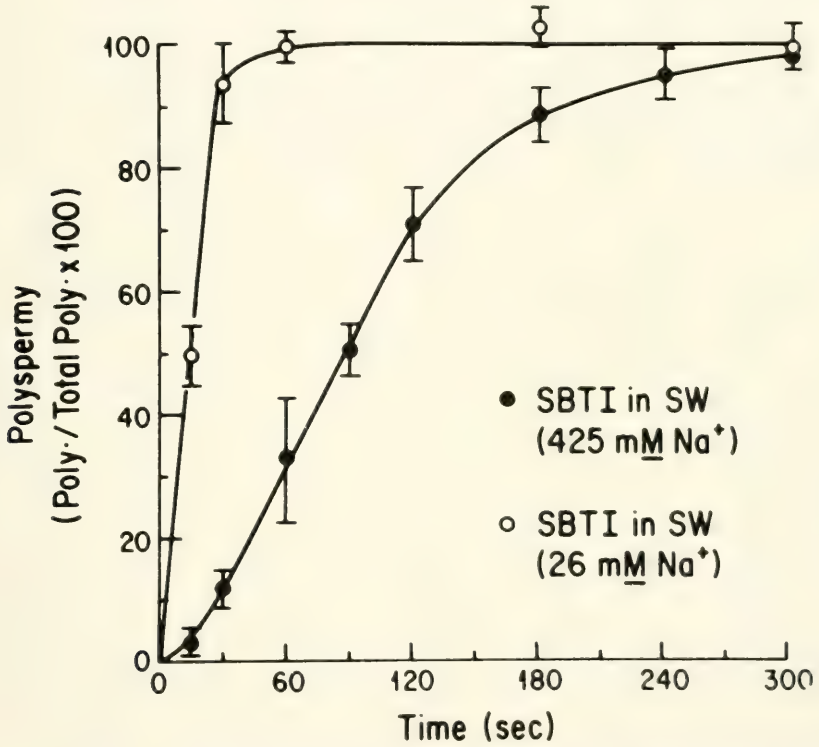


FIGURE 7. Kinetics of sodium-dependent block to polyspermy in *Arbacia punctulata* eggs as determined by the rate at which eggs become polyspermic in 2.5 ml SBTI (0.5 mg/ml) dissolved in natural (425 m*M* Na^+) and choline-substituted (26 m*M* Na^+) sea water. Eggs inseminated with excess (0.1 ml of 8%) sperm. Unfused sperm were killed at indicated times by addition of equal volume of sodium lauryl sulfonate (Hagstrom and Hagstrom, 1954), final concentration 0.0007%. Incidence of polyspermy estimated by counting multipolar divisions at first cleavage. Data normalized and expressed as percentage of all SBTI-treated eggs that were polyspermic in the absence of spermicide (91.3 ± 5.9 in 425 m*M* Na^+ and 71.7 ± 3.8 in 26 m*M* Na^+), minus the percentage of control eggs that were polyspermic in natural sea water alone (5.3 ± 2.1 in 425 m*M* Na^+ series and 7.0 ± 4.0 in 26 m*M* Na^+ series). n = 3. From Schuel and Schuel (1981), reprinted with permission of Academic Press, Inc.

when the oolemma that had initially depolarized to +20 mV is gradually repolarizing to the level (−70 mV) characteristic of the fertilized egg (Fig. 8). During normal fertilization in *Arbacia* elevation of the fertilization envelope (cortical reaction) is completed by 60 s after insemination, long before the rapid electrical (sodium dependent) block decays to 50% of its effectiveness at 90 s. Thus sea urchin eggs have adapted two typical cellular responses to stimulation, electrical depolarization of the plasma membrane and exocytotic release of stored secretory products, to operate in tandem to assure monospermic fertilization (Schuel and Schuel, 1981).

Presently available data suggests that the egg's fertilization potential regulates sperm-egg membrane fusion by blocking insertion/translocation into the oolemma of potential-sensitive components of the sperm's plasma membrane (Gould-Somero and Jaffe, 1984). Studies of cross species fertilization using voltage clamped sea urchin (*Strongylocentrotus purpuratus*) and *Urechis caupo* (a echiuroid worm) eggs showed that the blocking voltage for fertilization in these crosses is determined by the sperm species (Jaffe *et al.*, 1982). The electrical block to fertilization in *Lytechinus* eggs clamped at +15 mV can be overcome by brief repolarization to −60 mV for 35–45 ms (Shen and Steinhardt, 1984). These findings show that a sperm attached to the surface of an unfertilized egg can complete its electrically sensitive insertion/transfer process into the egg membrane within milliseconds. Finally, sperm can activate but will not penetrate *Lytechinus* eggs whose membranes are clamped at potentials more negative than −30 mV (Lynn and Chambers, 1984). The activating sperm is detached from the surface of these eggs as the fertilization envelope elevates. Hence, the oolemma

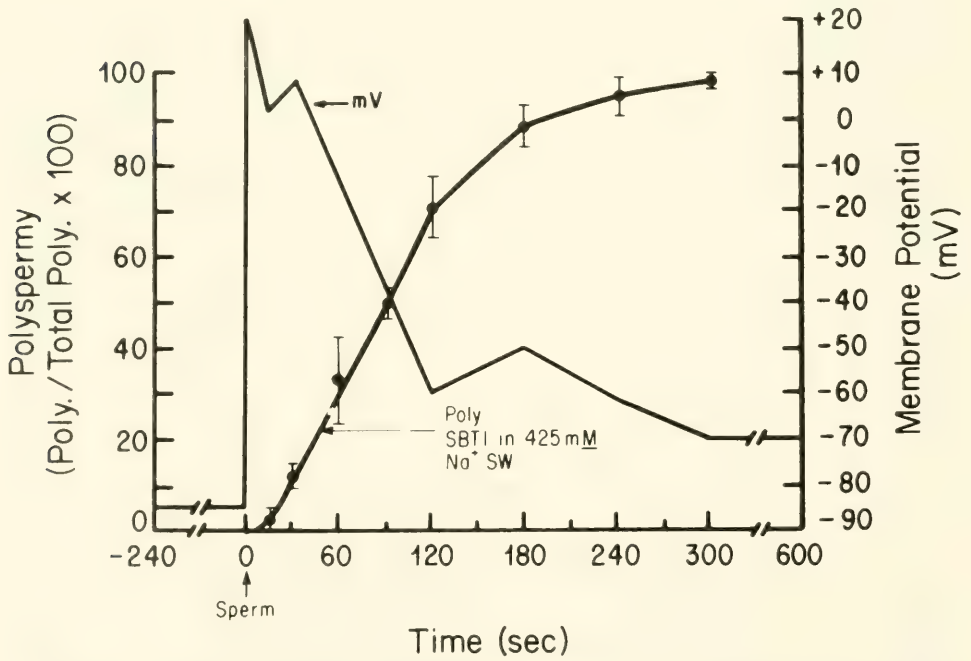


FIGURE 8. Decay of rapid block to polyspermy (normalized curve for polyspermy rate in SBTI-treated eggs in 425 m*M* Na^+ from Fig. 7) *versus* electrophysiological recording (provided by Jaffe and Tilney) of changes in membrane potential in an *Arbacia* egg during fertilization. From Schuel and Schuel (1981), reprinted with permission of Academic Press, Inc.

must partially depolarize after the sperm reacts with its receptor at the egg surface for membrane fusion to take place. These activated but unpenetrated eggs do not divide. They lack the sperm centriole required to organize the mitotic apparatus, and are comparable to parthenogenetically activated eggs that fail to cleave for the same reason (Lillie, 1919; Mazia, 1961; Schuel *et al.*, 1976b; Schuel, 1978). Together these findings indicate that the electric field across the egg's plasma membrane regulates sperm fusion and entry.

The existence of a rapid electrical block to polyspermy in sea urchins has been questioned (DeFelice and Dale, 1979; Dale and DeSantis, 1981; Dale *et al.*, 1982). This has generated considerable controversy in the literature (reviewed by: Dale and Monroy, 1981; Whitaker and Steinhardt, 1982; Shen, 1983; Nuccitelli and Grey, 1984; Schuel, 1984). According to Dale and his colleagues, the resting potential of physiologically normal (optimal) *Psammechinus* and *Paracentrotus* eggs varies from −8 to −16 mV. In their hands immature or aged eggs were reported to have resting potentials to −60 to −80 mV. Upon insemination of "optimal" eggs, the fertilizing sperm triggers a 1 to 2 mV step depolarization which precedes the main positive going depolarization (presumed to reflect propagation of the cortical reaction) by 13–15 s. Polyspermic eggs typically show additional step depolarizations (reflecting other successful sperm-egg fusion events) during this 13–15 s lag period. However, it should be noted that the fertilization potential changes recorded by Dale and his colleagues in what they consider to be "optimal" (physiologically normal) eggs are identical to what is seen in damaged eggs with leaky membranes (Chambers and DeArmendi, 1979). Such eggs lack the initial spike depolarization phase of the positive going fertilization potential (see Fig. 4, above).

Technical problems rather than species differences in polyspermy prevention probably account for the differences in experimental results reported by Jaffe (1976 and 1980) and by DeFelice and Dale (1979). The membrane of the sea urchin egg has a very high input resistance (Jaffe and Robinson, 1978; Chambers and DeArmendi, 1979) so that even minor leaks in the seal between the oolemma and a recording electrode can cause spuriously low readings in resting potential in the range of −10 mV (Nuccitelli and Grey, 1984). Independent data from studies which do not involve the potentially damaging effects of micro-electrode impalement of individual eggs are helpful in resolving the issue. Ion flux data confirm that the true resting potential of normal unfertilized eggs is about −70 mV (Jaffe and Robinson, 1978; Chambers and DeArmendi, 1979). The rapid spike depolarization seen in electrode-impaled eggs at fertilization (Jaffe, 1976; Chambers and DeArmendi, 1979; see Fig. 3, above) also can be recorded by the non-invasive measurement of action currents on the egg surface (Whitaker and Steinhardt, 1983). Suppression of the fertilization potential by low sodium sea water (Steinhardt *et al.*, 1971; Ito and Yoshioka, 1973; Chambers and DeArmendi, 1979) induces polyspermy in large populations of sea urchin eggs (Nishioka and Cross, 1978; Jaffe, 1980; Schuel and Schuel, 1981). Finally, the quality of the eggs used by DeFelice and Dale (1978) is questionable in view of the high incidence of polyspermy (70.1% avg., sperm density 2.4 to 21 $\times$ 10^7/ml) observed in the non-impaled eggs in their cultures. This may reflect damage to the eggs resulting from the acid sea water treatment used to remove the jelly coats (Hagstrom, 1956b, 1959). Consistent with this view, acid dejellied eggs have resting potentials initially of −8 to −15 mV which gradually change with standing in sea water to −60 to −80 mV (DeFelice and Dale, 1979; Taglietti, 1979). This might reflect recovery of the eggs from acid-induced damage. It would be of interest to test the capacity of these eggs to resist polyspermy. Approximately 85–95% monospermic fertilization should be obtained with good batches of eggs inseminated with 10^7 to 10^8 sperm/ml (Ginzburg,

1964; Boldt *et al.*, 1981; Schuel and Schuel, 1981; Alliegro and Schuel, 1984; Schuel *et al.*, 1984).

The weight of available evidence thus supports the operation of an ion-dependent electrical block to polyspermy in sea urchins as proposed by Jaffe (1976). It is probably functionally equivalent to the rapid block postulated by Rothschild and Swann (1952) in the sense that it is quickly established after the first successful sperm-egg reaction, is transiently active until the cortical reaction is completed, and reduces the probability that supernumerary sperm can penetrate the egg. Electrical blocks to polyspermy have been described in a variety of invertebrate and vertebrate eggs (reviewed by: Gould-Somero and Jaffe, 1984; Jaffe and Gould, 1984; Schuel, 1984).

Fertilization envelope

Observations of living sea urchin eggs during fertilization (see Fig. 2, above) led to the belief that the fertilization envelope acted as an absolute mechanical barrier to further sperm penetration (Fol, 1877; Hertwig and Hertwig, 1887). For the egg confronted by large numbers of excess sperm seeking entry, it provides an impregnable defense—a complete and continuous Maginot line. Sperm penetration ceases when elevation of the fertilization envelope is completed (Rothschild and Swann, 1952; Ginzburg, 1964; Schuel *et al.*, 1976b). If the elevated fertilization envelope is removed, subsequently added sperm can penetrate (re-fertilize) the egg to produce polyspermy (Sugiyama, 1951; Tyler *et al.*, 1956b; Longo, 1980, 1984).

The fertilization envelope is derived from the vitelline layer, a thin extracellular coat or glycocalyx, that is attached to the plasma membrane of the unfertilized egg. It is detached from the oolemma and transformed into the elevated and hardened fertilization envelope by the actions of products secreted by the cortical granules at fertilization (reviewed by: Epel, 1978; Schuel, 1978, 1984). This process is begun by the secreted cortical granule protease which cleaves peptide bonds linking the vitelline layer to the egg's plasma membrane (see *Cortical granule protease* section, below). The subsequent lifting of this detached investment (nascent fertilization envelope) is promoted by hydration and/or osmotic effects resulting from the secretion of sulfated acid mucopolysaccharides and other hydrophylic colloids into the developing perivitelline space by the discharging cortical granules (Schuel *et al.*, 1974; Schuel, 1978; Green and Summers, 1980). Immediately after its elevation (1–2 min post insemination) the fertilization envelope is easily removed by various mechanical or chemical treatments, but it gradually hardens over the next 10–30 min and becomes extremely difficult to remove or disperse (Runnstrom, 1966; Veron *et al.*, 1977; Schuel *et al.*, 1982b). Hardening is a complex process involving covalent cross-linking mediated by peroxidatic (Foerder and Shapiro, 1977; Hall, 1978) and possibly transpeptidation (Lallier, 1970) reactions, as well as structuralization by cortical granule derived structural proteins (reviewed by: Shapiro and Eddy, 1980; Schuel, 1978, 1984). Structuralization and cross-linking normally are completed by 10–15 min after insemination. During the next 15–20 min the permeability of the fertilization envelope to proteins is reduced greatly (Veron *et al.*, 1977). Collectively, these transformations act to protect the developing embryo from predation and other environmental hazards (Schuel, 1984).

As the fertilization envelope elevates, most of the sperm that initially were bound to the vitelline layer at the egg surface are detached (Vacquier and Payne, 1973). However, some sperm remain attached to the elevating or fully elevated fertilization envelope for an extended period of time, yet they can not penetrate it (Summers *et al.*, 1975; Summers and Hylander, 1976; Schuel and Schuel, 1981; also see Fig.

2, above). How are they excluded? One possibility is that hardening of the fertilization envelope makes it resistant to sperm penetration. However, sperm are unable to penetrate the soft fertilization envelope immediately after its elevation (Rothschild and Swann, 1952; Schuel *et al.*, 1976b) while structuralization and cross linking require 10–15 min for completion (Veron *et al.*, 1977; Schuel *et al.*, 1982b). Nicotine accelerates structuralization of the fertilization envelope (Longo and Anderson, 1970a). However, it has been reported that sperm can continue to enter nicotine-treated eggs to produce polyspermy after fertilization envelope is completely elevated (Hagstrom and Allen, 1957; Dale *et al.*, 1982). In addition, 3-amino-1,2,4-triazole inhibits the ovoperoxidase-catalyzed cross linking of the fertilization envelope during hardening (Foerder and Shapiro, 1977) but does not promote polyspermy (Boldt *et al.*, 1981; Coburn *et al.*, 1981). These findings suggest that either hardening is not involved in preventing polyspermy, or that sperm are excluded by a manifestation of hardening that cannot be detected by presently available experimental probes. Alternatively sperm may be inactivated by H_2O_2 that is produced during the cortical reaction (Boldt *et al.*, 1981; Coburn *et al.*, 1981) until some aspect of the hardening process renders the fertilization envelope completely resistant to sperm penetration. Future work should resolve these questions.

Cortical granule protease

A soybean trypsin inhibitor (SBTI) sensitive protease(s) is localized in cortical granules of unfertilized sea urchin eggs (Schuel *et al.*, 1973; Decker and Kinsey, 1983; Kopf *et al.*, 1983), and is secreted during the cortical reaction at fertilization or upon parthenogenetic activation (Vacquier *et al.*, 1973; Fodor *et al.*, 1975; Carroll, 1976; Schuel *et al.*, 1976b). This protease has been purified from unfertilized eggs and fertilization product (cortical granule exudate) by SBTI-affinity chromatography and characterized biochemically (Fodor *et al.*, 1975; Alliegro and Schuel, 1983, 1984; Sawada *et al.*, 1984). It is a serine protease that is trypsin like in its enzymatic properties.

The biological functions of the cortical granule protease in fertilization have been studied using SBTI and other serine protease inhibitors, and by the direct application of the purified protease to fertilization cultures (reviewed by: Epel, 1978; Schuel, 1978, 1984). The protease helps prevent polyspermy by promoting cortical granule exocytosis (Hagstrom, 1956a; Lonning, 1967; Longo and Schuel, 1973), by detaching the vitelline layer from the egg's plasma membrane to initiate elevation of the fertilization envelope (Longo and Schuel, 1973; Longo *et al.*, 1974; Carroll and Epel, 1975; Schuel *et al.*, 1976a), by proteolytic removal of sperm receptors in the vitelline layer (Aketa *et al.*, 1972; Vacquier *et al.*, 1973; Carroll, 1976; Glabe and Vacquier, 1978; Carroll *et al.*, 1982; Acevedo-Duncan and Carroll, 1983), and by promoting the generation of H_2O_2 by fertilized eggs (Coburn *et al.*, 1981). Limited proteolysis of egg surface proteins in the plasma membrane and/or vitelline layer takes place during fertilization (Shapiro, 1975; Ribot *et al.*, 1983). These processes may reflect multiple direct functions of the protease, a cascade reaction involving several proteases as in blood coagulation, and/or the activation of other enzymatic systems. Zymogen activation is a ubiquitous property of serine proteases in a wide variety of cellular systems, and produces a prompt and irreversible response to physiological stimuli to initiate new functions (Neurath and Walsh, 1976; Neurath, 1984), as occurs during fertilization (Schuel, 1978, 1984).

Eggs become polyspermic in SBTI because the cortical reaction is inhibited (Longo and Schuel, 1983; Vacquier *et al.*, 1973; Longo *et al.*, 1974; Schuel *et al.*, 1976). The

addition of equivalent amounts of bovine serum albumen, an inert protein, to the cultures does not affect the cortical reaction and does not cause polyspermy (Schuel and Schuel, 1981). SBTI is the most potent enzymatic inhibitor of the cortical granule protease known at present (Alliegro and Schuel, 1984). Furthermore, acid or alkali inactivation of SBTI (Kunitz, 1947) abolishes its capacity to inhibit this sea urchin egg protease *and* to cause polyspermy (Alliegro and Schuel, 1984). Other serine protease inhibitors (ovomucoid, limabean trypsin inhibitor, antipain, leupeptin, tosyl lysine chloromethyl ketone, benzamidine, etc.) mimic the effects of SBTI in producing polyspermy by inhibiting the cortical reaction (Hagstrom, 1956a; Runnstrom, 1966; Vacquier *et al.*, 1972a, b; Schuel *et al.*, 1973, 1976a, b; Hoshi *et al.*, 1979; Alliegro and Schuel, 1984; Sawada *et al.*, 1984). The biological effectiveness of trypsin inhibitors in promoting polyspermy shows a high correlation with their relative potencies as enzymatic inhibitors of the purified cortical granule protease (Table I). In view of these findings the claim by Dunham *et al.* (1982) that SBTI causes polyspermy because of a non-specific protein effect is unlikely to be correct.

SBTI-treated sea urchin eggs are monospermic initially (Hagstrom, 1956a) and polyspermy arises gradually by refertilization extending for a 15–20 min period after the initial insemination (Hagstrom, 1956a; Longo and Schuel, 1973). This process was elucidated in SBTI-treated *Arbacia* eggs by: (1) determining the receptivity of monospermic eggs (obtained by minimal initial insemination) to refertilization upon subsequent re-insemination with excess sperm; combined with (2) a morphometric analysis (transmission electron micrographs) of the cortical reaction (Longo *et al.*, 1973; Schuel *et al.*, 1976a). The results of these studies are depicted schematically in Figure 9. In control eggs the cortical reaction (transition from Fig. 9A–C) normally is completed by 60 s after insemination but requires 15–20 min for completion in SBTI-treated eggs. Cortical granule exocytosis is completed in SBTI-treated eggs by 3 min post insemination (Fig. 9B). At this time the vitelline layer has detached only over regions formerly occupied by the discharged cortical granules, but remains attached to the plasma membrane at numerous sites (cortical projections) that were devoid of subjacent cortical granules prior to fertilization. These regions correspond to the functional sperm penetration sites because receptivity of the SBTI-treated egg to polyspermy (refertilization) at 3 min is the same as that of the unfertilized egg. Receptivity to refertilization only declines coincidentally with the gradual detachment

TABLE I

Comparison of the potencies of serine protease inhibitors in inhibiting the purified cortical granule protease and promoting polyspermy in sea urchins

Inhibitor	ID_{50} (*M*)	P_{50} (*M*)
Soybean trypsin inhibitor	6.01×10^{-9}	4.94×10^{-6}
Ovomucoid	1.06×10^{-6}	1.21×10^{-4}
Limabean trypsin inhibitor	3.47×10^{-6}	1.42×10^{-4}
Leupeptin	4.22×10^{-6}	8.48×10^{-4}
Antipain	3.16×10^{-5}	3.69×10^{-4}

ID_{50}: Inhibitor conc. to inhibit 50% of enzyme activity using benzoyl-1-arginine ethyl ester as substrate. Protease purified from extract of unfertilized *Strongylocentrotus purpuratus* eggs by SBTI-affinity chromatography.

P_{50}: Inhibitor conc. to promote polyspermy in 50% of *Arbacia punctulata* eggs inseminated with excess sperm.

According to Spearman's rank correlation, $r_s = 0.90$.

From Alliegro and Schuel (1984), reprinted with permission of *Biol. Bull.*

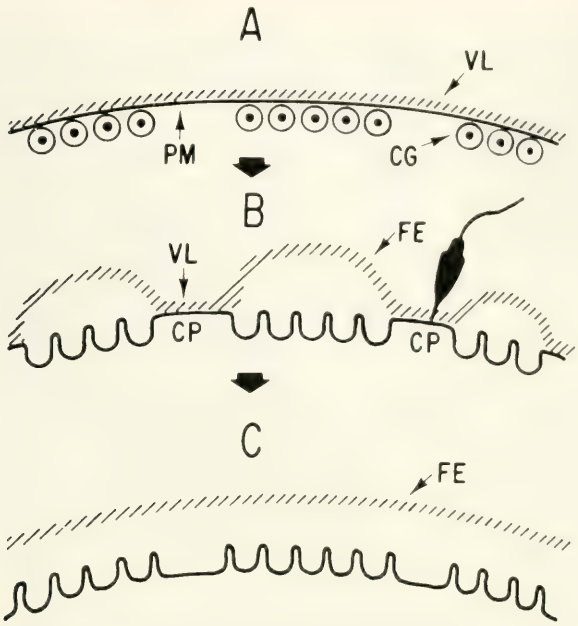


FIGURE 9. Schematic diagram depicting the effects of SBTI in causing polyspermy and inhibiting the cortical reaction in sea urchin eggs. The transition from A to C is normally completed in control *Arbacia punctulata* eggs within 60 s after insemination. From Schuel (1978), reprinted with permission of Alan R. Liss, Inc. *A:* Surface of an unfertilized egg showing regions where cortical granules (CG) are tightly packed under the plasma membrane (PM) interspersed with randomly distributed areas devoid of subjacent cortical granules. The vitelline layer (VL) is attached to the outer surface of the egg's plasma membrane (PM). Transition from A to B is sensitive to inhibition of SBTI. *B:* Surface of SBTI-treated egg at 3 min after insemination. The fertilization envelope has elevated over regions previously occupied by the discharged cortical granules. The vitelline layer remains attached to the egg's plasma membrane at the apex of the cortical projections. These sites correspond to regions where the plasma membrane was devoid of subjacent cortical granules before fertilization. Sperm can continue to fuse with and penetrate the egg at these sites as long as they are available. Transition from B to C sensitive to inhibition by SBTI. *C:* Surface of SBTI-treated egg at 20 min after insemination. The fertilization envelope has elevated from the entire surface of the egg and prevents the entrance of additional sperm.

of the vitelline layer from the cortical projections over the next 15–20 min (Fig. 9C). Sperm penetration can continue at these sites in SBTI-treated eggs as long as they are available (Fig. 10). Detachment of the vitelline layer from these potential penetration sites is required to complete the cortical block to polyspermy. Refertilization (polyspermy) also is facilitated because SBTI-treated eggs can not produce H_2O_2 to inactivate sperm at their surfaces (Coburn *et al.*, 1981). However, SBTI-treated eggs do not fill up with supernumerary sperm under these conditions because the electrical (sodium dependent) block is still operative and tends to limit sperm penetration (Schuel and Schuel, 1981).

Large numbers of sperm rapidly bind to the vitelline layer at insemination, and most of them subsequently detach from the elevating fertilization envelope (Vacquier and Payne, 1973; Summers and Hylander, 1976). Sperm detachment is prevented by SBTI (Fig. 11). Application of the cortical granule protease (Carroll and Epel, 1975; Carroll, 1976) or bovine trypsin (Aketa *et al.*, 1972) to unfertilized eggs prevents the binding of subsequently added sperm. These findings suggest that the cortical granule protease removes sperm receptors from the vitelline layer (Glabe and Vacquier,

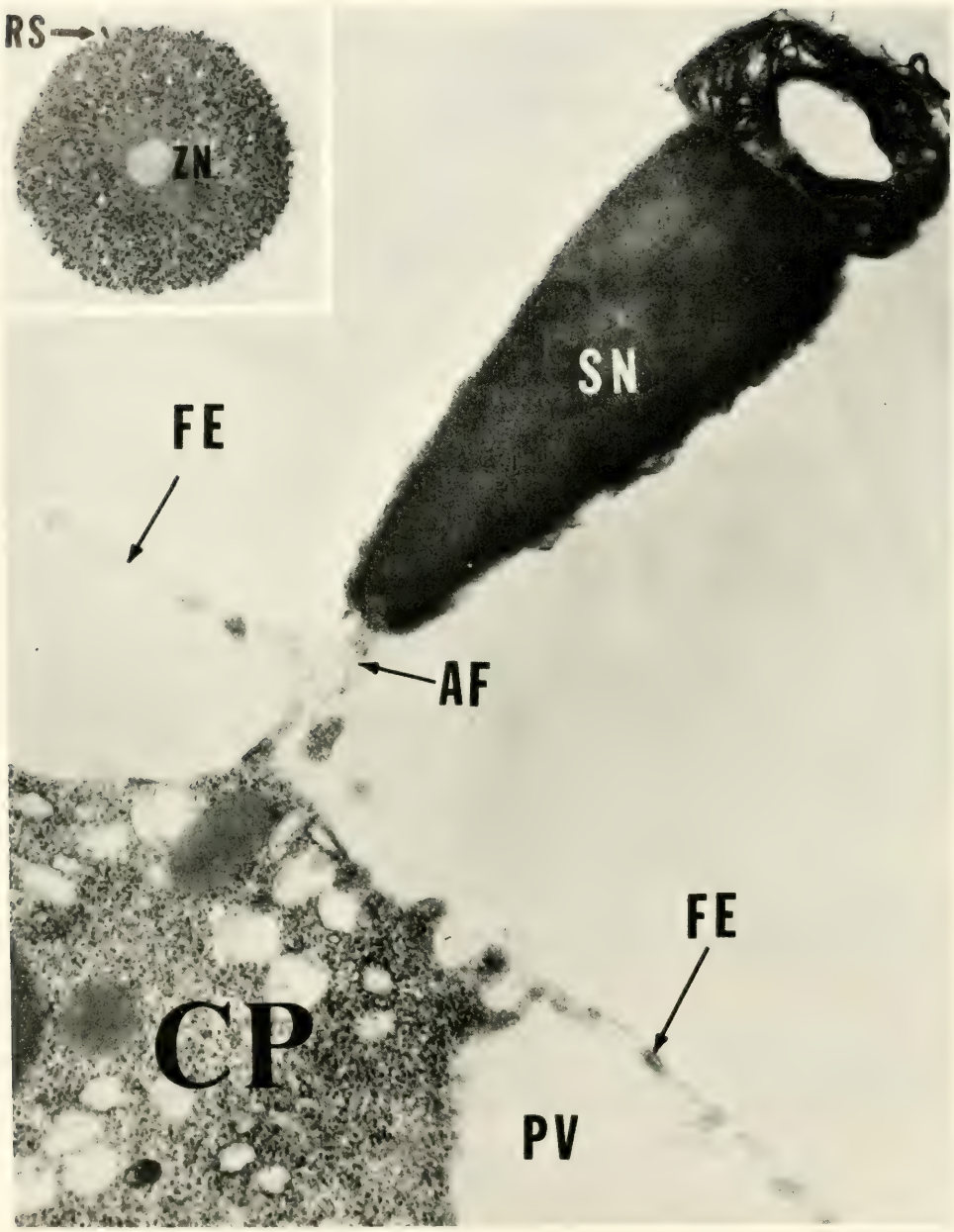


FIGURE 10. Spermatozoon associated with apical surface of a cortical projection (CP) via its acrosomal process (AF) in SBTI-treated *Abracia* zygote seven minutes after initial minimal insemination, fixed 2 min after subsequent re-insemination with excess sperm. Fertilization envelope (FE), perivitelline space (PV), sperm nucleus (SN). 37,800×. Inset: Photomicrograph of zygote re-inseminated 12 min after initial insemination. Zygote nucleus (ZN), refertilizing spermatozoon (RS). 450×. From Longo *et al.* (1974), reprinted with permission of Academic Press, Inc.

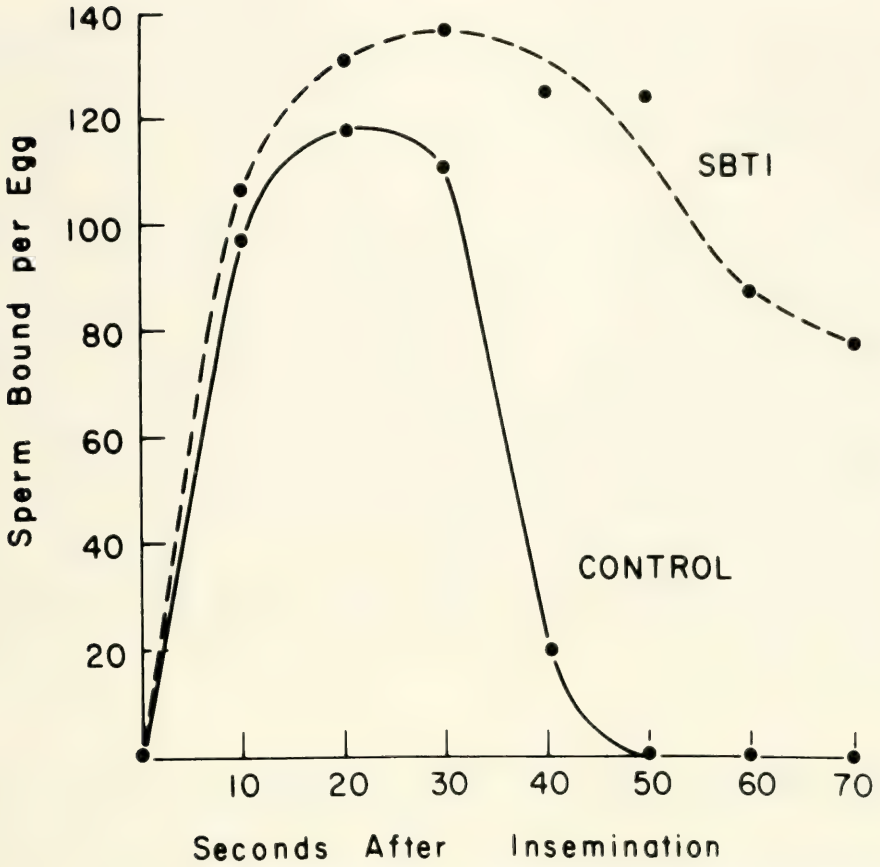


FIGURE 11. Effect of SBTI on sperm binding and detachment during fertilization in *Strongylocentrotus purpuratus*. Eggs were inseminated in sea water (● —— ●) or 1 mg/ml SBTI (● - - - ●), and the number of sperm bound per egg was determined at indicated times after insemination. Sperm rapidly bind to the egg surfaces and then begin to detach as the cortical reaction propagates around the surface of the eggs (compare with Fig. 2, above). This process is completed in control eggs within 50 s. SBTI-treated eggs do not show the normal detachment phase. From Vacquier *et al.* (1973), reprinted with permission of Academic Press.

1978; Yoshida and Aketa, 1978). This process tends to reduce the probability of polyspermy. Detachment of the vitelline layer and destruction of sperm receptors in sea urchins may be promoted by two distinct SBTI-sensitive proteases that are secreted by the cortical granules (Carroll and Epel, 1975; Carroll, 1976; Carroll *et al.*, 1982). Similar phenomena apparently operate in mammalian eggs where the secretion of a "trypsin-like" cortical granule protease has been implicated in altering the zona pellucida to prevent polyspermy (Gwatkin *et al.*, 1973; Bleil *et al.*, 1981; Wolf, 1981).

Peroxide mediated block

Phagocytic leukocytes employ peroxidatic reactions involving secreted myeloperoxidase and H_2O_2 to kill bacteria and other microorganisms (Klebanoff, 1980). The peroxidatic system activated in sea urchin eggs at fertilization, secreted ovoperoxidase and H_2O_2 which promotes cross-linking of the fertilization envelope, was

postulated to also act as a spermicidal agent to help prevent polyspermy (Foerder and Shapiro, 1977; Klebanoff *et al.*, 1979; Shapiro and Eddy, 1980), since H_2O_2 is toxic to sperm (Evans, 1947). Evidence for a peroxide-mediated block to polyspermy in sea urchins has been obtained (Boldt *et al.*, 1981; Coburn *et al.*, 1981). Sperm are rapidly inactivated by egg-derived H_2O_2. However, a peroxidase endogenous to the sperm appears to be responsible for their inactivation (Boldt, 1982; Boldt *et al.*, 1981, 1984).

Sea urchin eggs release H_2O_2 into the ambient sea water during the cortical reaction at fertilization (Fig. 12). The fertilizing capacity of sperm is rapidly reduced by treatment with equivalent concentrations of H_2O_2 (Boldt *et al.*, 1981; Coburn *et al.*, 1981). For example, with minimal sperm densities, 300 μM H_2O_2 produces an instantaneous 35% reduction in sperm fertility which is further suppressed to a 90% reduction over five minutes (Boldt *et al.*, 1981). The fertilizing capacity of control sperm in sea water remains constant during this period. Removal of egg-derived H_2O_2 by adding catalase to fertilization cultures of *Arbacia* produces a concentration dependent induction of polyspermy (Coburn *et al.*, 1981; Dunham *et al.*, 1982). Catalase does not impair elevation of the fertilization envelope. It must be added prior to completion of the cortical reaction to cause polyspermy (Fig. 13). The addition of equivalent amounts of bovine serum albumin, a non-enzymatic protein, does not cause polyspermy (Coburn *et al.*, 1981; Schuel, 1984). Furthermore, inactivation of catalase by heating or addition of 3-amino-1,2,4-triazole, an inhibitor which binds irreversibly with the catalase-H_2O_2

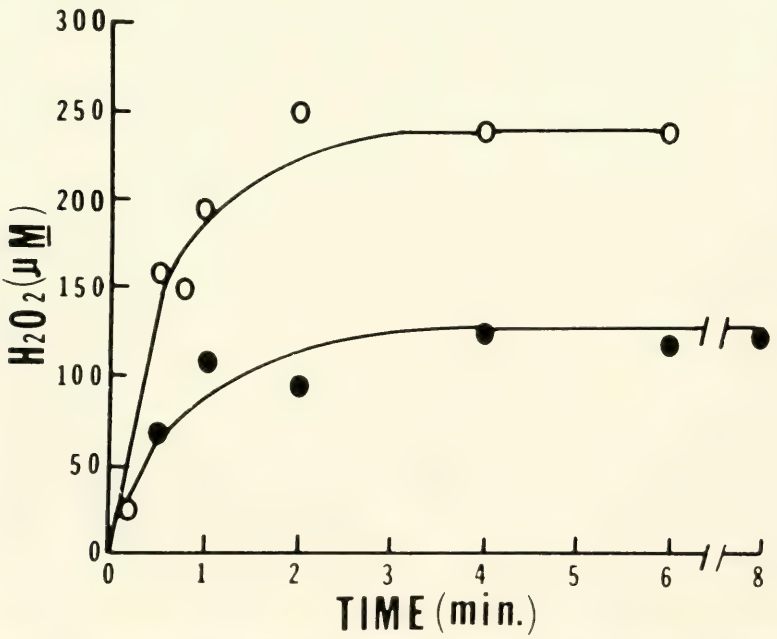


FIGURE 12. Release of H_2O_2 by *Strongylocentrotus purpuratus* eggs during fertilization. Eggs inseminated with a sperm density just sufficient to obtain 100% fertilization. Gametes were removed from the cultures at indicated times after insemination. The concentration of H_2O_2 was determined in the supernatants by the horseradish peroxidase mediated oxidation of pyrogallol; – ○ – ○ –, 0.2 ml packed eggs/10 ml culture; – ● – ● –, 0.1 ml packed eggs/10 ml culture. From Boldt *et al.* (1981), reprinted with permission of Alan R. Liss, Inc.

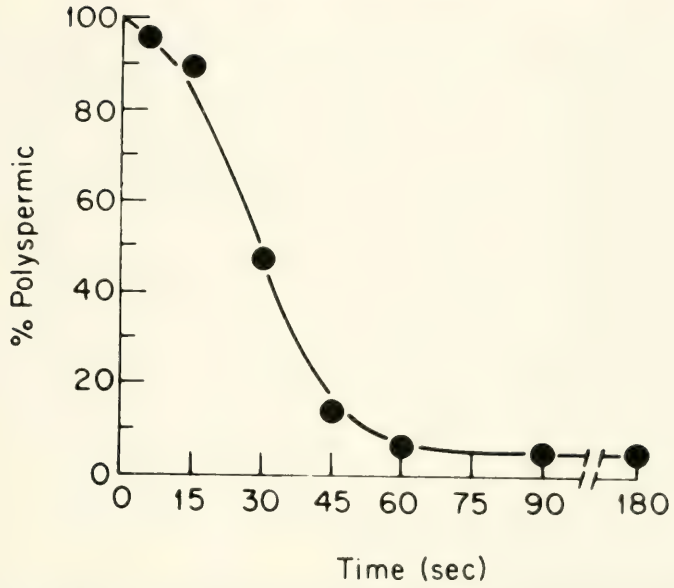


FIGURE 13. Timing of catalase induced polyspermy in *Arbacia punctulata* eggs. Catalase (2 mg/ml final conc.) added to cultures at indicated times after insemination with excess sperm. When catalase is added after the cortical reaction is completed at 60 s post insemination, the incidence of polyspermy is the same as in control eggs fertilized in sea water. From Coburn *et al.* (1981), reprinted with permission of Academic Press.

complex (Margoliash and Novogrodsky, 1960), abolishes its capacity to promote polyspermy (Coburn *et al.*, 1981). Together these findings show that: (1) sea urchin eggs produce H_2O_2 to help prevent polyspermy until the cortical reaction is completed; and (2) catalase promotes polyspermy by the enzymatic removal of egg-derived H_2O_2. The claim that catalase causes polyspermy because of a non-specific protein effect (Dunham *et al.*, 1982) is unlikely to be correct.

The putative role of ovoperoxidase in preventing polyspermy was examined using phenylhydrazine and 3-amino-1,2,4-triazole (Boldt *et al.*, 1981). These are the two most potent inhibitors of ovoperoxidase in *Strongylocentrotus* (Foerder and Shapiro, 1977), and *both* would be expected to cause polyspermy if ovoperoxidase were involved. Phenylhydrazine promotes polyspermy while 3-amino-1,2,4-triazole does not (Boldt *et al.*, 1981). These results suggested that the egg's ovoperoxidase does not have a functional role in preventing polyspermy, and implied that a peroxidase endogenous to the sperm might be involved.

Evidence for such a sperm peroxidase has been obtained (Boldt *et al.*, 1981, 1984). Phenylhydrazine protects sperm from inactivation by H_2O_2, while 3-amino-1,2,4-triazole potentiates the adverse effect of H_2O_2 on sperm fertility (Boldt *et al.*, 1981). Phenylhydrazine is a well known peroxidase inhibitor (Hidaka and Udenfriend, 1970), while 3-amino-1,2,4-triazole is a classic catalase inhibitor (Margoliash and Novogrodsky, 1960). Biochemical studies have shown that sea urchin sperm actually contain a peroxidase that is preferentially inhibited by phenylhydrazine and a 3-amino-1,2,4-triazole sensitive catalase (Boldt, 1982; Boldt and Schuel, 1982; Boldt *et al.*, 1984).

These data suggest that sperm fertility is modulated by reactions of their endogenous catalase and peroxidase with egg-derived H_2O_2 during fertilization. Hydrogen peroxide

is a potent cytotoxic substance (Klebanoff, 1980). Catalase is a ubiquitous enzyme in aerobic cells and protects them by removing H_2O_2 generated during normal oxidative metabolism (Chance *et al.*, 1979). The sperm catalase probably performs a similar function (Rothschild, 1950). It also appears to protect sperm from H_2O_2 produced by fertilized eggs (Boldt *et al.*, 1981; Coburn *et al.*, 1981). Peroxidases use H_2O_2 to oxidize other substances. The products of such reactions can be cytotoxic (Klebanoff, 1980). The sperm peroxidase may perform an analogous function by utilizing H_2O_2 released by fertilized eggs to reduce sperm fertility, and thus assist in preventing polyspermy.

As in phagocytic cells, peroxide production by eggs during fertilization is associated with a burst in cyanide insensitive oxygen consumption (Foerder *et al.*, 1978; Klebanoff *et al.*, 1979; Klebanoff, 1980; Perry and Epel, 1981). Two thirds of the oxygen consumed by *Strongylocentrotus* eggs at fertilization is converted into H_2O_2 (Foerder *et al.*, 1978). Superoxide anion is produced along with H_2O_2 during the respiratory burst by phagocytes. Together these oxygen species can generate other extremely cytotoxic oxygen radicals via the Haber-Weiss reaction (Klebanoff, 1980). Chemiluminescence detected during fertilization (Foerder *et al.*, 1978) may reflect the formation of such oxygen radicals. The cytotoxic effects of phagocytes on microorganisms and tumor cells can be reduced by addition of catalase and/or superoxide dismutase to the cultures (Kuehl and Egan, 1980; Fridovich, 1982; Halliwell, 1982; Nathan, 1982). Similar processes may operate in sea urchin gametes at fertilization since added catalase or superoxide dismutase promote polyspermy (Coburn *et al.*, 1981; Dunham *et al.*, 1982).

The respiratory burst and H_2O_2 production may be linked to other early events in eggs at fertilization such as the release of calcium from intracellular stores, the influx of external sodium ions, and cortical granule secretion (Steinhardt and Epel, 1974; Foerder *et al.*, 1978; Whitaker and Steinhardt, 1982). The respiratory burst in sea urchin eggs is blocked in sodium-free sea water (Whitaker and Steinhardt, 1982). This finding suggests that there may be a functional link between the sodium dependent (Jaffe, 1980; Schuel and Schuel, 1981) and peroxide mediated (Boldt *et al.*, 1981; Coburn *et al.*, 1981) blocks to polyspermy. A similar situation may apply to the functions of the cortical granule protease. SBTI is a potent enzymatic inhibitor of the cortical granule protease (see *Cortical granule protease* section, above), and it also blocks the production of H_2O_2 by fertilized eggs (Coburn *et al.*, 1981). This may account, at least in part, for the high levels of polyspermy seen in SBTI-treated sea urchin eggs. SBTI and other serine protease inhibitors prevent the production of oxygen radicals by phagocytes (Janoff and Carp, 1982; Troll *et al.*, 1982). This again is suggestive of the many biochemical similarities between sea urchin gametes and somatic phagocytes.

Does a peroxide mediated block to polyspermy operate in mammals? Studies intended to answer this question have yet to be conducted, but there are several highly suggestive similarities between sea urchins and mammals. Cortical granule derived ovoperoxidase is involved in cross-linking the fertilization envelope in sea urchins (Foerder and Shapiro, 1977; Hall, 1978) and the zona pellucida in mammalian eggs (Gulyas and Schmell, 1980; Schmell and Gulyas, 1980). Given this situation, the fertilized mammalian egg would be expected to produce H_2O_2 required for cross-linking as well. Furthermore, H_2O_2 and other active oxygen metabolites are very toxic to mammalian sperm (Smith and Klebanoff, 1970; Jones and Mann, 1973; Holland *et al.*, 1982; Alvarez and Storey, 1982, 1983), just as they are to sea urchin sperm (Evans, 1947; Boldt *et al.*, 1981). These phenomena in mammalian gametes should be studied in relation to polyspermy prevention.

Arachidonic acid cascade

Changes in phospholipid metabolism occur in sea urchin eggs during fertilization (Schmell and Lennarz, 1974; Barber and Mead, 1975; Byrd, 1975; Kozhina *et al.*, 1978; Barber, 1979; Turner *et al.*, 1983). Membrane fusion between secretory granules and the plasma membrane is promoted by the action of a calcium-activated phospholipase A_2 in both somatic secretory cells (Laychock and Putney, 1982; Rubin, 1982) as well as sea urchin eggs (Ferguson and Shen, 1984) and sperm (Conway and Metz, 1976; SeGall and Lennarz, 1981). Phospholipase A_2 releases a free fatty acid (usually arachidonic acid) and lysophosphoglycerides from membrane phosphoglycerides (Fig. 14). These substances may be re-incorporated into membrane phospholipids via reacylation reactions in sea urchin eggs during fertilization (Schmell and Lennarz, 1974; Turner *et al.*, 1983) or in secreting somatic cells (Laychock and Putney, 1982; Rubin, 1982). Lysophosphoglycerides can initiate exocytosis by promoting membrane fusion (Lucy, 1970; Laychock and Putney, 1982). Free arachidonic acid can be oxidized by two major pathways (Kuehl and Egan, 1980). The cyclooxygenase pathway leads to the production of prostaglandins and thromboxanes. Non-steroidal anti-inflammatory drugs (NSAID) act by inhibiting cyclooxygenase (Flower, 1974; Kuehl and Egan, 1980). The lipoxygenase pathway leads to the production of the leukotrienes and hydroxy fatty acids (HETE's). Cells may contain different lipoxygenases which oxidize the carbon atom at positions 5, 11, 12, or 15 of arachidonic acid (Nelson *et al.*, 1982; Samuelsson, 1983). Leukotrienes are products of the 5-lipoxygenase pathway. The slow reacting substance of anaphylaxis (SRS-A) consists of leukotrienes LTC_4,

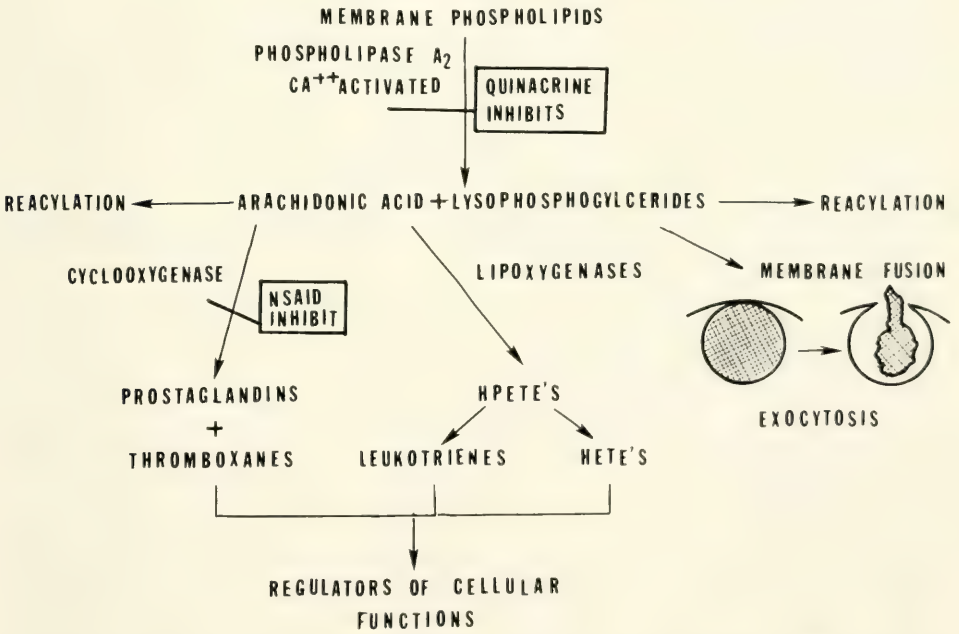


FIGURE 14. Schematic diagram depicting role of phospholipase A_2 in the initiation of the arachidonic acid cascade and the membrane fusion reaction in exocytosis, as discussed in text. Non-steroidal anti-inflammatory drugs (NSAID), hydroperoxy eicosatetraenoic acids (HPETE'S), hydroxy eicosatetraenoic acids (HETE'S).

LTD_4, and LTE_4 (Samuelsson, 1983). Products derived from the enzymatic oxidation of arachidonic acid by cyclooxygenase and lipoxygenases are widely distributed in invertebrates and vertebrates (Flower, 1974; Nomura and Ogata, 1976; Morse *et al.*, 1977), are extremely potent mediators of inflammatory reactions and also are modulators of a wide variety of normal cellular functions in somatic tissues (Kuehl and Egan, 1980; Laychock and Putney, 1982; Nelson *et al.*, 1982; Samuelsson, 1983).

Several lines of evidence suggest a role for phospholipase A_2 in triggering cortical granule discharge in sea urchin eggs at fertilization. The cortical reaction can be induced by treating unfertilized eggs with melittin which is a phospholipase A_2 activator from bee venom (Ohman, 1945; Shimada *et al.*, 1982). Similar results can be obtained by the application of lysolecithin (Ohman, 1945; Schuel, 1978), a potent fusigen liberated from membrane lipids by phospholipase (Lucy, 1970). Quinacrine inhibits phospholipase A_2 in blood platelets (Lapetina *et al.*, 1981). Unfertilized sea urchin eggs contain a calcium activated phospholipase A_2 that is sensitive to inhibition by quinacrine (Ferguson and Shen, 1984). Furthermore, quinacrine inhibits the cortical reaction and promotes polyspermy in fertilization cultures (Ferguson and Shen, 1984).

Evidence has been obtained implicating arachidonic acid oxidation in preventing polyspermy (Schuel *et al.*, 1983; Schuel *et al.*, 1984). Arachidonic acid is located at the second acyl position of phosphoglycerides and is released by the action of phospholipase A_2 (Hill and Lands, 1970; Lapetina *et al.*, 1981; Bach, 1982; Laychock and Putney, 1982). Membrane phospholipids of cortical granules and plasma membrane in unfertilized sea urchin eggs contain abundant (about 17% of total fatty acids) arachidonic acid (Decker and Kisney, 1983). Arachidonic acid is liberated from phospholipids in eggs at fertilization by a calcium activated phospholipase (Perry, 1979), presumably phospholipase A_2 (Ferguson and Shen, 1984). The synthesis of arachidonic acid oxidation products of the cyclooxygenase (prostaglandins) and cyclooxygenase (12-HETE-like molecule) pathways at fertilization has been reported (Perry, 1979). Enzyme inhibitors and specific product antagonists have been used as probes to show a role for these reactions in the block to polyspermy. Indomethacin, a NSAID that is a potent cyclooxygenase inhibitor in somatic tissues (Flower, 1974; Kuehl and Egan, 1980), causes a dose and sperm density dependent induction of polyspermy if added before the egg completes the cortical reaction (Schuel *et al.*, 1984). Indomethacin does not retard the cortical reaction (Elhai, 1981; Schuel *et al.*, 1984), and does not promote polyspermy by protecting sperm from peroxidatic inactivation by egg-derived H_2O_2 (Schuel *et al.*, 1984). Other potent cyclooxygenase inhibitors (flufenamate and meclofenamate) also cause polyspermy (Table II). FPL-55712, an antagonist for leukotrienes LTC_4 and LTD_4 (Sheard *et al.*, 1982), causes a dose and sperm density dependent induction of polyspermy (P_{50} = 2.5 ± 0.8 μM in *Arbacia* at 4.0 ± 2.2 sperm/ml, n = 6) if added before the eggs complete the cortical reaction (Moss *et al.*, 1983). To determine which gamete is affected by FPL-55712, eggs and sperm were pretreated with the drug which was removed by dilution at fertilization. Sperm pretreated with FPL-55712 do not cause polyspermy in control eggs. However, eggs pretreated with FPL-55712 become heavily polyspermic upon insemination with control sperm. These findings suggest that reaction of leukotrienes with receptors on the egg's surface modulate its receptivity to sperm during the cortical reaction. BW755C, a potent inhibitor of the 5-lipoxygenase involved in leukotriene synthesis during inflammatory reactions (Higgs and Mugridge, 1983), also causes polyspermy in sea urchins (Schuel and Schuel, unpubl. data). Hence products derived from both the cyclooxygenase and 5-lipoxygenase pathways for oxidation of arachidonic acid appear to modulate gamete interaction and help prevent polyspermy in sea urchins.

TABLE II

Relative potency of NSAID in promoting polyspermy in Stronglyocentrotus

Drug	P_{50}* (μM)
Indomethacin (n = 8)	38.1 ± 18.4
Flufenamic Acid (n = 8)	20.6 ± 10.5
Meclofenamate (n = 4)	16.9 ± 9.4

* P_{50}: Concentration of drug at which 50% of the eggs were polyspermic.

Incidence of polyspermy in control eggs; 14.7 ± 11.8%. Sperm density: 9.8 ± 4.5 × 10^7/ml. n = 13.

Aspirin, a weak cyclooxygenase inhibitor, did not cause polyspermy at conc. up to 5 mM (data not shown).

From Schuel *et al.* (1984), reprinted with permission of Alan R. Liss, Inc.

The metabolism of arachidonic acid in other systems is regulated by the amount of substrate (free arachidonic acid) available for oxidation (Vogt, 1978; Racowsky and Biggers, 1983). If this were true for sea urchin eggs as well, then addition of exogenous arachidonic acid to unfertilized eggs should result in the formation of products that inhibit fertilization. Added arachidonic acid has been reported to reduce the fertility of sea urchin eggs (Elhai and Scandella, 1983).

Additional work is required to identify directly the arachidonic acid products that are produced during fertilization and to determine how they act to prevent polyspermy. Similar processes may operate in other animals as well. For example, the mechanism by which molluscan eggs prevent polyspermy has been an enigma because there is no overt structural change at the egg surface comparable to the cortical reaction in sea urchins (Longo, 1973; Alliegro and Wright, 1983). However, molluscan eggs are a rich source of cyclooxygenase (Morse *et al.*, 1977). Do they use cyclooxygenase metabolites to prevent polyspermy? Arachidonic acid derived products also may regulate cortical granule secretion (Elhai, 1981) and other aspects of fertilization. An ionophore-like role for lipoxygenase products in releasing calcium from internal stores during egg activation has been proposed (Epel *et al.*, 1982). Both cyclooxygenase and lipoxygenase products are known to exhibit properties of calcium ionophores in somatic tissues (Laychock and Putney, 1982; Weissmann *et al.*, 1982). The acrosome reaction in human sperm involves a membrane associated phospholipase A_2 (Thakkar *et al.*, 1984) and appears to be regulated by arachidonic acid oxidation products (Meizel and Turner, 1983). In view of these observations it is tempting to speculate that arachidonic acid derived metabolites also may help prevent polyspermy in mammals.

There may be a functional link between the arachidonic acid cascade and the peroxidatic block to polyspermy in sea urchins (see *Peroxide mediated block* section, above) since: (1) cytotoxic oxidizing radicals are produced during peroxidatic reactions in the arachidonic acid cascade (Kuehl and Egan, 1980; Kuehl *et al.*, 1982); (2) both H_2O_2 and superoxide anion are required to initiate the arachidonic acid cascade (Kuehl and Egan, 1980; Fridovich, 1982); and (3) H_2O_2 can have a role in the metabolism of prostaglandins (Paredes and Weiss, 1982). There also may be a link between the functions of the cortical granule protease and the arachidonic acid cascade since thrombin treatment activates phospholipases and arachidonic acid oxidation in platelets (Lapetina *et al.*, 1981). In addition, serine protease inhibitors block the stimulus-induced release of arachidonic acid by platelets (Feinstein *et al.*, 1980).

Hyaline layer

A role in polyspermy prevention has been attributed to the hyaline layer (reviewed by: Rothschild, 1956; Allen, 1957; Runnstrom *et al.*, 1959; Tyler and Tyler, 1966b; Epel, 1978; Schuel, 1978, 1984). This concept is based upon findings that the hyaline layer restricts sperm penetration into eggs that are experimentally denuded of their fertilization envelopes (Ishida and Nakano, 1950; Sugiyama, 1951; Hagstrom and Hagstrom, 1954b; Nakano, 1956; Tyler *et al.*, 1956b; Longo, 1980). Such demembranated eggs can be refertilized, but the frequency of polyspermy declines with time (Tyler *et al.*, 1956b; Schatten, 1978; Longo, 1980). The reduced receptivity of these eggs to refertilization is caused by the formation of the hyaline layer which gradually covers up sperm receptors in the egg's plasma membrane (Tyler and Tyler, 1956b). Removal of this barrier by dispersing the hyaline layer with calcium-magnesium free sea water makes it possible to refertilize zygotes or embryos through the 8-cell stage of development (Sugiyama, 1951; Runnstrom *et al.*, 1959; Longo, 1980 and 1984).

Demembranated eggs gradually become refractory to sperm penetration even after the hyaline layer is removed (Schatten, 1978; Longo, 1980). This effect was attributed to the establishment of a late permanent block to polyspermy at the level of the egg's plasma membrane (Schatten, 1978). This interpretation may not be correct. Hyalin, the major structural protein of the hyaline layer, is stored in cortical granules of unfertilized eggs (reviewed by: Schuel, 1978, 1984). The hyaline layer that is formed during fertilization is derived from this source. However, hyalin also is stored in small cytoplasmic vesicles of unfertilized eggs that do not secrete during fertilization (Hylander and Summers, 1982) and which normally discharge during subsequent embryonic development (Kane, 1973). This secondary reservoir is probably the source of hyalin protein that can regenerate the hyaline layer after its experimental removal (Citkowitz, 1971; Kane, 1973; Schuel *et al.*, 1982a). Such regeneration of the hyaline layer probably accounts for the gradual reduction in receptivity of demembranated eggs to refertilization (polyspermy) after the initially formed hyaline layer is removed (Longo, 1980).

The hyaline layer is unlikely to be a significant factor in preventing polyspermy during normal fertilization because sperm can not penetrate either the elevating or fully elevated fertilization envelope (Rothschild and Swann, 1952; Rothschild, 1956; Schuel, 1978, 1984; also see *Fertilization envelope* section, above). Another possibility is that the protein hyalin also might impregnate the fertilization envelope and help render it resistant to sperm penetration. However, immuno-electron cytochemical studies using mono-specific antibodies against pure hyalin did not detect hyalin in the fertilization envelope (Hylander and Summers, 1982). Nevertheless, the hyaline layer may serve as a final line of defense against polyspermy for an egg that has lost its fertilization envelope because of an environmental insult in nature, or experimental manipulation in the laboratory.

Sulfated acid mucopolysaccharides

Cortical granules of unfertilized sea urchin eggs contain sulfated acid mucopolysaccharides (SAMP) that are secreted during the cortical reaction at fertilization (Ishihara, 1968; Bal, 1970; Schuel *et al.*, 1974). These acid polyanions are thought to participate in the lifting of the fertilization envelope by promoting the influx of water into the developing perivitelline space (Schuel *et al.*, 1974; Schuel, 1978).

The cytochemical localization of SAMP in the cortical granules was facilitated by using quaternary ammonium salts to prevent loss of SAMP during fixation (Schuel *et al.*, 1974). The natural ionic environment of sea urchin eggs in sea water favors

the formation of stable complexes between SAMP and quaternary ammonium salts (Kelly *et al.*, 1963). Brief exposure of unfertilized eggs to several quaternary ammonium salts (cetyltrimethyl ammonium bromide, cetylpyridinium chloride and bromide, tetraethylammonium chloride, etc.) produces residual adverse effects on subsequent fertilization in sea water in terms of reduced fertility and greatly increased vulnerability to polyspermy in eggs that do fertilize (Schuel *et al.*, 1974). Eggs cannot be fertilized in the presence of the quaternary ammonium salts because these substances inactivate and/or kill the sperm. These findings suggest that cortical granule derived SAMP may have a role in preventing polyspermy (Schuel *et al.*, 1974). However, the quaternary ammonium salts could produce polyspermy by different mechanisms since some of them may alter the egg's plasma membrane by acting as detergents or otherwise modifying its electrical properties (Schuel *et al.*, 1974; Schuel, 1978; Shen, 1983). This phenomenon should be studied further.

The Nicotine Controversy

Nicotine has been recognized to be a polyspermy promoting agent for almost a century (Hertwig and Hertwig, 1887; Clark, 1936). However, the mechanism by which nicotine causes polyspermy remains controversial because the drug appears to produce multiple effects on gamete interactions, and because observations made by various investigators are not consistent with each other. This may reflect species differences in response to nicotine, and/or differences in experimental procedures used by various investigators.

Binding of sperm to the egg surface is greatly enhanced in nicotine-treated eggs, especially if the jelly coat had been removed prior to insemination (Hagstrom and Allen, 1956; Longo and Anderson, 1970a). Sperm remain bound to the elevated fertilization envelope in nicotine-treated eggs. Thus nicotine promotes a more stable adhesion of the gametes which could allow more sperm to fuse with the egg than normal (Longo and Anderson, 1970a).

Nicotine inhibits the rapid block to polyspermy (Rothschild, 1956). Nicotine-treated eggs may fertilize more rapidly than controls (Rothschild and Swann, 1950; Rothschild, 1953; Dale *et al.*, 1982), although contrary results have been reported (Hagstrom and Allen, 1956). Sperm do not swim more rapidly in nicotine so sperm motility can not account for the higher fertilization rate (Rothschild and Swann, 1950; Rothschild, 1953). Nicotine also affects the electrical properties of the egg's plasma membrane as reflected by decreased membrane resistance, altered current voltage relation such that outward current produces a smaller shift in potential, and reduced amplitude of the fertilization potential (Jaffe, 1980; Dale *et al.*, 1982). These findings show that nicotine inhibits the electrical block to polyspermy (Jaffe, 1980). Dale's group (1982) claim that these electrical phenomena are not related to polyspermy prevention (see *Electrical block* section, above).

Another view holds that nicotine produces polyspermy by inhibiting some aspect of the cortical reaction (Hagstrom and Allen, 1956; Dale *et al.*, 1982). Elevation of the fertilization envelope in nicotine-treated eggs usually is completed at the same time as in controls (Rothschild, 1953; Hagstrom and Allen, 1956; Longo and Anderson, 1970a). In some cases it elevates more rapidly in nicotine because cortical granule discharge is initiated at multiple loci which correspond to the sites where supernumerary sperm fuse with the egg (Rothschild and Swann, 1950; Longo and Anderson, 1970a). Sperm may continue to enter nicotine-treated eggs *after* elevation of the fertilization envelope has been completed (Hagstrom and Allen, 1956; Dale *et al.*, 1982). If correct, this finding suggests that nicotine may inhibit *the* transformation in the fertilization

envelope that is responsible for excluding sperm. Impaired structuralization of the fertilization envelope can not be responsible for continued sperm penetration because nicotine accelerates structuralization (Longo and Anderson, 1970a). However, other workers found that sperm incorporation ceases in nicotine-treated eggs when elevation of the fertilization envelope is completed (Baker and Presley, 1966). Cortical granule secretion occasionally is arrested by nicotine so that the fertilization envelope elevates only over a restricted portion of the egg surface (Hagstrom and Allen, 1956; Hulser and Schatten, 1982). Another possibility is that nicotine may act by inhibiting the cortical granule protease (Carroll, 1976).

How nicotine produces polyspermy remains enigmatic. Multiple actions of the drug on gamete binding, fertilization potential, cortical granule secretion, and the fertilization envelope may be involved. Additional work under standardized conditions is required to clarify this question.

Conclusions

Sea urchin eggs show an extraordinary capacity to resist polyspermy upon insemination with excess sperm. Research summarized above has revealed that this is due to the redundancy of defense mechanisms all of which act together to help assure monospermic fertilization. Factors associated with external reproduction in the ocean must have exerted tremendous selective pressures for the development of these multiple polyspermy preventing mechanisms because a great excess of sperm is required to insure that fertilization would take place. Several of these polyspermy preventing mechanisms have been conserved in mammals (Gwatkin, 1977; Schuel, 1978, 1984; Wolf, 1981; Schmell *et al.*, 1983) where an excess of sperm may arrive at the site of fertilization within the female's reproductive tract.

Heretofore it has been assumed that the responsibility for polyspermy prevention resided exclusively with the fertilized egg. The sole function attributed to the sperm during fertilization was to try to enter the egg. However, functional cooperation by both interacting gametes appears to be involved in preventing polyspermy. Sperm are active participants in both the electrical (Jaffe *et al.*, 1982) and peroxide mediated (Boldt *et al.*, 1981, 1984) blocks. They are pre-programmed to reduce their fertility in response to electrical and chemical signals produced by the fertilized egg. Natural selection and evolution have fashioned an exquisite set of cellular responses and regulatory processes that are elicited during gamete interaction to increase the probability of normal monospermic fertilization. Within this context, it is not an individual spermatozoon but rather the production of a diploid zygote that is important for successful reproduction and continuation of the species.

Fertilization is a cellular process. Typical responses to stimulation that are common to all cells (electrical depolarization of the plasma membrane, calcium-triggered release of secretory products via exocytosis, limited proteolysis, respiratory burst, release of H_2O_2, and generation of arachidonic acid oxidation products, etc.) have been adapted by the interacting gametes to help prevent polyspermy. For somatic secretory cells such as those involved in inflammatory reactions, these responses are initiated by the reaction of a specific ligand with its receptor in the cell's plasma membrane. The equivalent function is subserved during fertilization by the reaction of complementary molecular determinants on the surface of the egg and the sperm at the site of gamete fusion. In this sense, fertilization and polyspermy prevention can be considered to be akin biochemically to an inflammatory reaction in somatic tissues.

Multiple polyspermy preventing processes have been identified in sea urchins. Additional work is required for us to understand the precise mechanisms by which

they operate. Other polyspermy preventing processes may still await discovery. These apparently multiple blocks may turn out to be steps in one or a few associated metabolic pathways. The variety of agents capable of causing polyspermy may act at different points in a sequence of cellular reactions, and possibly interrupt the synthesis of specific product(s) critical to polyspermy prevention. Elucidation of these phenomena in sea urchins should provide insights that are useful in other areas of developmental, reproductive, and cellular biology.

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LITERATURE CITED

ACEVEDO-DUNCAN, M. E., AND E. J. CARROLL. 1983. Isolation of a sperm receptor polypeptide from the sea urchin egg vitelline layer. *J. Cell Biol.* **97:** 181a.

AKETA, K., K. ONITAKE, AND H. TSUZUKI. 1972. Tryptic disruption of sperm-binding site of sea urchin egg surface. *Exp. Cell Res.* **71:** 27–32.

ALLEN, R. D. 1957. The initiation of development. Pp. 17–67 in *Chemical Basis of Development*, W. D. McElroy, and B. Glass, eds. Johns Hopkins University Press, Baltimore, MD.

ALLIEGRO, M. C., AND H. SCHUEL. 1983. Further characterization of protease isolated from unfertilized sea urchin eggs. *J. Cell Biol.* **97:** 180a.

ALLIEGRO, M. C., AND H. SCHUEL. 1984. Specificity in the induction of polyspermy in sea urchin eggs by soybean trypsin inhibitor. *Biol. Bull.* **166:** 473–481.

ALLIEGRO, M. C., AND D. A. WRIGHT. 1983. Polyspermy inhibition in the oyster, *Crassostrea virginica*. *J. Exp. Zool.* **227:** 127–137.

ALVAREZ, J. G., AND B. T. STOREY. 1982. Spontaneous lipid peroxidation in rabbit epididymal spermatozoa. Its effect on sperm motility. *Biol. Reprod.* **27:** 1102–1108.

ALVAREZ, J. G., AND B. T. STOREY. 1983. Role of superoxide dismutase in protecting rabbit spermatozoa from O_2 toxicity due to lipid peroxidation. *Biol. Reprod.* **28:** 1129–1136.

ANDERSON, E. 1968. Oocyte differentiation in the sea urchin, *Arbacia punctulata*, with particular reference to the cortical granules and their participation in the cortical reaction. *J. Cell Biol.* **37:** 514–539.

AZARNIA, R., AND E. L. CHAMBERS. 1976. The role of divalent cations in activation of the sea urchin egg. I. Effect of fertilization on divalent cation content. *J. Exp. Zool.* **198:** 65–78.

BACH, M. K. 1982. Mediators of anaphylaxis and inflammation. *Ann. Rev. Microbiol.* **36:** 371–413.

BAKER, P. F., AND R. PRESLEY. 1966. A direct method of measuring the rate of sperm entry into sea urchin eggs. *J. Physiol.* **186:** 47P–49P.

BAL, A. K. 1970. Selective staining of ultrastructural components of cortical granules and Golgi cisternae of sea urchin eggs. *Z. Zellforsch.* **104:** 471–477.

BARBER, M. L. 1979. Changes in enzyme activities and lipid content of echinoderm egg membranes at maturation and fertilization. *Am. Zool.* **19:** 821–837.

BARBER, M. L., AND J. F. MEAD. 1975. Comparison of lipids of sea urchin egg ghosts prepared before and after fertilization. *Wilhelm Roux' Archiv* **177:** 19–27.

BIBRING, T., J. BAXANDALL, AND C. C. HARTER. 1984. Sodium-dependent pH regulation in active sea urchin sperm. *Dev. Biol.* **101:** 425–435.

BLEIL, J. D., C. F. BEALL, AND P. M. WASSERMAN. 1981. Mammalian sperm-egg interaction: fertilization of mouse eggs triggers modification of the major zone pellucida glycoprotein, ZP_2. *Dev. Biol.* **86:** 189–197.

BOLDT, J. 1982. The role of hydrogen peroxide in the block to polyspermy during sea urchin fertilization. Ph.D. Thesis, State University of New York, Buffalo.

BOLDT, J., AND H. SCHUEL. 1982. Biochemical studies on sperm catalase and peroxidase that modulate sperm fertility during fertilization in the sea urchin. *J. Cell Biol.* **95:** 150a.

BOLDT, J., H. SCHUEL, R. SCHUEL, P. V. DANDEKAR, AND W. TROLL. 1981. Reaction of sperm with egg-derived hydrogen peroxide helps prevent polyspermy during fertilization in the sea urchin. *Gamete Res.* **4:** 365–377.

BOLDT, J., M. ALLIEGRO, AND H. SCHUEL. 1984. A separate catalase and peroxidase in sea urchin sperm. *Gamete Res.* (in press).

BOOLOOTIAN, R. A. 1966. Reproductive physiology. Pp. 561–613 in *Physiology of Echinodermata*, R. A. Boolootian, ed. J. Wiley and Sons, NY.

BYRD, E. W. 1975. Phospholipid metabolism following fertilization in sea urchin eggs and embryos. *Dev. Biol.* **46:** 309–316.

BYRD, E. W., AND F. D. COLLINS. 1975. Absence of fast block to polyspermy in eggs of sea urchin *Strongylocentrotus purpuratus*. *Nature* **257:** 675–677.

CARDASIS, C., AND H. SCHUEL. 1976. The sea urchin egg as a model system to study effects of narcotics on secretion. Pp. 631–640 in *Tissue Responses to Addictive Drugs,* D. H. Ford and D. Clouet, eds. Spectrum Publ., NY.

CARROLL, E. J. 1976. Cortical granule proteases from sea urchin eggs. *Methods Enzymol.* **45:** 343–353.

CARROLL, E. J., AND D. EPEL. 1975. Isolation and biological activity of the proteases released by sea urchin eggs following fertilization. *Dev. Biol.* **44:** 22–32.

CARROLL, E. J., A. F. LOIS, AND D. A. LACKEY. 1982. Physiochemical characterization of sperm receptor hydrolase from sea urchin eggs. *J. Cell Biol.* **95:** 154a.

CHAMBERS, E. L., AND J. DEARMENDI. 1979. Membrane potential, action potential and activation potential of eggs of the sea urchin, *Lytechinus variegatus*. *Exp. Cell Res.* **122:** 203–218.

CHANCE, B., H. SIES, AND A. BOVERIS. 1979. Hydroperoxide metabolism in mammalian organs. *Physiol. Rev.* **59:** 527–605.

CHASE, D. G. 1967. Inhibition of the cortical reaction with high hydrostatic pressure and its effects on the fertilization and early development of sea urchin eggs. Ph.D. Thesis, University of Washington.

CHRISTEN, R., R. W. SCHACKMAN, AND B. M. SHAPIRO. 1983. Interactions between sperm and sea urchin egg jelly. *Dev. Biol.* **98:** 1–14.

CITKOWITZ, E. 1971. The hyaline layer: its isolation and role in echinoderm development. *Dev. Biol.* **24:** 348–362.

CLARK, J. M. 1936. An experimental study of polyspermy. *Biol. Bull.* **70:** 361–384.

COBURN, M., H. SCHUEL, AND W. TROLL. 1981. A hydrogen peroxide block to polyspermy in the sea urchin *Arbacia punctulata*. *Dev. Biol.* **84:** 235–238.

COLWIN, L. H., AND A. L. COLWIN. 1967. Membrane fusion in relation to sperm-egg association. Pp. 295–367 in *Fertilization* Vol. 1, C. B. Metz and A. Monroy, eds. Academic Press, NY.

CONWAY, A. F., AND C. B. METZ. 1976. Phospholipase activity of sea urchin sperm: its possible involvement in membrane fusion. *J. Exp. Zool.* **198:** 39–48.

DALE, B., AND A. DESANTIS. 1981. Maturation and fertilization of the sea urchin oocyte: an electrophysiological study. *Dev. Biol.* **85:** 474–484.

DALE, B., AND A. MONROY. 1981. How is polyspermy prevented? *Gamete Res.* **4:** 151–169.

DALE, B., A. DESANTIS, AND B. E. HAGSTROM. 1982. The effect of nicotine on sperm-egg interaction in the sea urchin: polyspermy and electrical events. *Gamete Res.* **5:** 125–135.

DAN, J. C. 1967. Acrosome reaction and lysins. Pp. 237–293 in *Fertilization* Vol. 1, C. B. Metz and A. Monroy, eds. Academic Press, NY.

DECKER, G. L., AND W. J. LENNARZ. 1979. Sperm binding and fertilization envelope formation in a cell surface complex isolated from sea urchin eggs. *J. Cell Biol.* **81:** 92–103.

DECKER, S. J., AND W. H. KINSEY. 1983. Characterization of cortical secretory vesicles from the sea urchin egg. *Dev. Biol.* **96:** 37–45.

DEFELICE, L. J., AND B. DALE. 1979. Voltage response to fertilization and polyspermy in sea urchin eggs and oocytes. *Dev. Biol.* **72:** 327–341.

DUNHAM, P., L. NELSON, L. VOSSHALL, AND G. WEISSMANN. 1982. Effects of enzymatic and non-enzymatic proteins on *Arbacia* spermatozoa: reactivation of aged sperm and the induction of polyspermy. *Biol. Bull.* **163:** 420–430.

ELHAI, J. 1981. Arachidonic acid and other fatty acids: Signal transmission in the fertilized sea urchin egg. Ph.D. Thesis, State University of New York, Stony Brook.

ELHAI, J., AND C. J. SCANDELLA. 1983. Arachidonic acid and other fatty acids inhibit secretion from sea urchin eggs. *Exp. Cell Res.* **148:** 63–71.

EPEL, D. 1978. Mechanisms of activation of sperm and egg during fertilization of sea urchin gametes. *Curr. Topics Dev. Biol.* **12:** 186–246.

EPEL, D., G. PERRY, AND T. SCHMIDT. 1982. Intracellular calcium and fertilization: role of the cation and regulation of intracellular calcium levels. Pp. 171–183 in *Membranes in Growth and Development,* J. F. Hoffman, G. H. Giebisch, and L. Bolis, eds. Alan R. Liss, Inc., NY.

EVANS, T. C. 1947. Effects of hydrogen peroxide produced in the medium by irradiation on spermatozoa of *Arbacia punctulata*. *Biol. Bull.* **92:** 99–109.

FEINSTEIN, M. B., J. Y. VANDERHOEK, AND R. WALENGA. 1980. Serine-esterase (protease) inhibitors block stimulus-induced mobilization of arachidonate in platelets. Pp. 321–325 in *Advances in Prostaglandin and Thromboxane Research* Vol. 6, B. Samuelsson, P. W. Ramwell, and R. Paoletti, eds. Raven Press, NY.

FERGUSON, J. E., AND S. S. SHEN. 1984. Evidence of phospholipase A_2 in the sea urchin egg: its possible involvement in the cortical reaction. *Gamete Res.* **9:** 329–338.

FLOWER, R. J. 1974. Drugs that inhibit prostaglandin biosynthesis. *Pharm. Rev.* **26:** 33–67.

FODOR, E. J. B., H. AKO, AND K. A. WALSH. 1975. Isolation of a protease from sea urchin eggs before and after fertilization. *Biochemistry* **14:** 4923–4927.

FOERDER, C. A., AND B. M. SHAPIRO. 1977. Release of ovoperoxidase from sea urchin eggs hardens the fertilization membrane with tyrosine cross-links. *Proc. Natl. Acad. Sci.* **74:** 4214–4218.

FOERDER, C. A., S. J. KLEBANOFF, AND B. M. SHAPIRO. 1978. Hydrogen peroxide production, chemiluminescence, and the respiratory burst of fertilization: interrelated events in early sea urchin development. *Proc. Natl. Acad. Sci.* **75:** 3183–3187.

FOL, H. 1877. Sur le commencement de l'hénogenie chez divers animaux. *Arch. Zool. Exp. Gén.* **T-6:** 145–169.

FRIDOVICH, I. 1982. Superoxide dismutase in biology and medicine. Pp. 1–19 in *Pathology of Oxygen*, A. P. Autor, ed. Academic Press, NY.

GACHE, C., H. L. NIMAN, AND V. D. VACQUIER. 1983. Monoclonal antibodies to the sea urchin egg vitelline layer inhibit fertilization by blocking sperm adhesion. *Exp. Cell Res.* **147:** 63–74.

GINZBURG, A. S. 1964. Mechanism of blockade of polyspermia in echinoderms. *Dokl. Biol. Sci. S.S.S.R.* **152:** 1232–1235.

GINZBURG, A. S. 1972. *Fertilization in Fishes and the Problem of Polyspermy.* Israel Program Scientific Translations, Ltd., Jerusalem. 366 pp.

GLABE, C. G., AND W. J. LENNARZ. 1981. Isolation of a high molecular weight glycoconjugate derived from the surface of *Strongylocentrotus purpuratus* eggs that is implicated in sperm adhesion. *J. Supramol. Struct. Cell Biochem.* **15:** 387–394.

GLABE, C. G., AND V. D. VACQUIER. 1977. Isolation and characterization of the vitelline layer of sea urchin eggs. *J. Cell Biol.* **75:** 410–421.

GLABE, C. G., AND V. D. VACQUIER. 1978. Egg surface glycoprotein receptor for sea urchin sperm bindin. *Proc. Natl. Acad. Sci.* **75:** 881–885.

GOULD-SOMERO, M., AND L. A. JAFFE. 1984. Control of cell fusion at fertilization by membrane potential. Pp. 27–38 in *Cell Fusion: Gene Transfer and Transformation*, R. F. Beers and E. G. Bassett, eds. Raven Press, NY.

GREEN, J. D. 1983. Sperm bind but do not unbind from the fixed sea urchin egg. *Dev. Growth Differ.* **25:** 315–321.

GREEN, J. D., AND R. G. SUMMERS. 1980. Formation of the cortical concavity at fertilization in the sea urchin egg. *Dev. Growth Differ.* **22:** 821–829.

GREEN, J. D., AND R. G. SUMMERS. 1982. Effects of protease inhibitors on sperm-related events in sea urchin fertilization. *Dev. Biol.* **93:** 139–144.

GULYAS, B. J., AND E. D. SCHMELL. 1980. Ovoperoxidase activity in ionophore treated mouse eggs. I. Electron microscopic localization. *Gamete Res.* **3:** 267–277.

GWATKIN, R. B. L. 1977. *Fertilization Mechanisms in Man and Mammals.* Plenum Press, NY. 161 pp.

GWATKIN, R. B. L., D. T. WILLIAMS, J. F. HARTMANN, AND M. KNIAZUK. 1973. The zona reaction of hamster and mouse eggs: production *in vitro* by a trypsin-like protease from cortical granules. *J. Reprod. Fert.* **32:** 259–265.

HAGIWARA, S., AND L. A. JAFFE. 1979. Electrical properties of egg cell membranes. *Ann. Rev. Biophys. Bioeng.* **8:** 385–416.

HAGSTROM, B. E. 1956a. Studies on polyspermy in sea urchins. *Arkiv. Zool.* **10:** 307–315.

HAGSTROM, B. E. 1956b. The effect of removal of the jelly coat on fertilization in sea urchins. *Exp. Cell Res.* **10:** 740–743.

HAGSTROM, B. E. 1959. Further studies on jelly-free sea urchin eggs. *Exp. Cell Res.* **17:** 256–261.

HAGSTROM, B. E., AND R. D. ALLEN. 1956. The mechanism of nicotine-induced polyspermy. *Exp. Cell Res.* **10:** 14–23.

HAGSTROM, B., AND B. HAGSTROM. 1954a. A method of determining the fertilization rate in sea urchins. *Exp. Cell Res.* **6:** 479–484.

HAGSTROM, B., AND B. E. HAGSTROM. 1954b. Re-fertilization of the sea urchin egg. *Exp. Cell Res.* **6:** 491–496.

HALL, H. G. 1978. Hardening of the fertilization envelope by peroxidase-catalyzed phenolic coupling of tyrosines. *Cell* **15:** 343–355.

HALLIWELL, B. 1982. Production of superoxide, hydrogen peroxide, and hydroxyl radicals by phagocytic cells: a cause of chronic inflammatory disease. *Cell Biol. Internat. Rep.* **6:** 529–542.

HARRISON, P. L., R. C. BABCOCK, G. D. BULL, J. K. OLIVER, C. C. WALLACE, AND B. L. WILLIS. 1984. Mass spawning in tropical reef corals. *Science* **223:** 1186–1188.

HARVEY, E. B. 1956. *The American Arbacia and Other Sea Urchins.* Princeton Univ. Press, Princeton, NJ. 298 pp.

HEILBRUNN, L. V. 1956. *Dynamics of Living Protoplasm.* Academic Press, NY. 327 pp.

HERTWIG, O., AND R. HERTWIG. 1887. Uber die Befruchtungs und Theilungsvorgange des thierischen Eies unter den Einfluss ausserer Agentien. *Jen. Zeitschr. Naturw.* **20:** 120–241 and 477–510.

HIDAKA, H., AND S. UDENFRIEND. 1970. Evidence of a hydrazine-reactive group at the active site of the nonheme portion of horseradish peroxidase. *Arch. Biochem. Biophys.* **140:** 174–180.

HIGGS, G. A., AND K. G. MUGRIDGE. 1983. Inhibition of mononuclear leukocyte accumulation by the arachidonic acid lipoxygenase inhibitor BW755C. Pp. 19–23 in *Advances in Prostaglandin, Thromboxane and Leukotriene Research,* Vol. 12., B. Samuelsson, R. Paoletti, and P. Ramwell, eds. Raven Press, NY.

HILL, E. E., AND W. E. M. LANDS. 1970. Phospholipid metabolism. Pp. 185–257 in *Lipid Metabolism,* S. J. Waki, ed. Academic Press, NY.

HOLLAND, M. D., J. G. ALVAREZ, AND B. T. STOREY. 1982. Production of superoxide and activity of superoxide dismutase in rabbit epididymal spermatozoa. *Biol. Reprod.* **27:** 1109–1118.

HOSHI, M., AND T. MORIYA. 1980. Arylsulfatase of sea urchin sperm. 2. Arylsulfatases as a lysin of sea urchins. *Dev. Biol.* **74:** 343–350.

HOSHI, M., T. MORIYA, T. AOYAGI, H. UMEZAWA, H. MOHRI, AND Y. NAGAI. 1979. Effects of hydrolase inhibitors on fertilization of sea urchins: I. Protease inhibitors. *Gamete Res.* **2:**107–119.

HULSER, D., AND G. SCHATTEN. 1982. Bioelectric responses at fertilization: separation of the events associated with insemination from those due to the cortical reaction in the sea urchin, *Lytechinus variegatus. Gamete Res.* **5:** 363–377.

HYLANDER, B. L., AND R. G. SUMMERS. 1982. An ultrastructural immunocytochemical localization of hyalin in the sea urchin egg. *Dev. Biol.* **93:** 368–380.

ISHIDA, J., AND E. NAKANO. 1950. Fertilization of activated sea urchin eggs deprived of fertilization membrane by washing with Ca-Mg-free media. *Annot. Zool. Japn.* **23:** 43–48.

ISHIHARA, K. 1968. An analysis of acid polysaccharides produced at fertilization of sea urchin. *Exp. Cell Res.* **51:** 473–484.

ITO, S., AND K. YOSHIOKA. 1973. Effect of various ionic compositions upon the membrane potentials during activation of sea urchin eggs. *Exp. Cell Res.* **78:** 191–200.

JAFFE, L. A. 1976. Fast block to polyspermy in sea urchin eggs is electrically mediated. *Nature* **261:** 68–71.

JAFFE, L. A. 1980. Electrical polyspermy block in sea urchins: nicotine and low sodium experiments. *Dev. Growth Differ.* **22:** 503–507.

JAFFE, L. A., AND M. GOULD. 1984. Polyspermy-preventing mechanisms. In *Biology of Fertilization,* C. B. Metz and A. Monroy, eds. Academic Press, NY., in press.

JAFFE, L. A., AND K. R. ROBINSON. 1978. Membrane potential of the unfertilized sea urchin egg. *Dev. Biol.* **62:** 215–218.

JAFFE, L. A., M. GOULD-SOMERO, AND L. Z. HOLLAND. 1982. Studies of the mechanism of the electrical polyspermy block using voltage clamp during cross-species fertilization. *J. Cell Biol.* **92:** 616–621.

JAFFE, L. F. 1980. Calcium explosions as triggers of development. *Ann. NY. Acad. Sci.* **339:** 86–101.

JANOFF, A., AND H. CARP. 1982. Proteases, antiproteases, oxidants: pathways of tissue injury during inflammation. Pp. 62–82 in *Current Topics in Inflammation and Infection,* G. Majno, R. S. Cotran, and N. Kaufman, eds. Williams and Wilkins, Baltimore.

JONES, R., AND T. MANN. 1973. Lipid peroxidation in spermatozoa. *Proc. R. Soc. Lond. (Biol.)* **184:** 103–107.

JUST, E. E. 1919. The fertilization reaction in *Echinarachnius parma.* I. Cortical response of the egg to insemination. *Biol. Bull.* **36:** 1–10.

JUST, E. E. 1939. *The Biology of the Cell Surface.* P. Blakiston's Son and Co., Philadelphia, PA. 392 pp.

KANE, R. E. 1973. Hyaline release during normal sea urchin development and its replacement after removal at fertilization. *Exp. Cell Res.* **81:** 301–311.

KELLY, J. W., G. D. BLOOM, AND J. SCOTT. 1963. Quaternary ammonium compounds in connective tissue histochemistry: I. selective unblocking. *J. Histochem. Cytochem.* **11:** 791–798.

KILLIE, R. A. 1959. Surface changes in gametic cells. *Proc. R. Phys. Soc. Edinb.* **28:** 101–106.

KINSEY, W. H., AND W. J. LENNARZ. 1981. Isolation of a glycopeptide fraction from the surface of the sea urchin egg that inhibits sperm-egg binding and fertilization. *J. Cell Biol.* **91:** 325–331.

KINSEY, W. H., G. K. SEGALL, AND W. J. LENNARZ. 1979. The effect of the acrosome reaction on the respiratory activity and fertilizing capacity of echinoid sperm. *Dev. Biol.* **71:** 49–59.

KLEBANOFF, S. J. 1980. Oxygen metabolism and the toxic properties of phagocytes. *Ann. Int. Med.* **93:** 480–489.

KLEBANOFF, S. J., C. A. FOERDER, E. M. EDDY, AND B. M. SHAPIRO. 1979. Metabolic similarities between fertilization and phagocytosis. *J. Exp. Med.* **149:** 938–953.

KOZHINA, V. P., T. A. TEREKHOVA, AND V. I. SVETASHEV. 1978. Lipid composition of gametes and embryos of the sea urchin *Strongylocentrotus intermedius* at early stages of development. *Dev. Biol.* **62:** 512–517.

KOPF, G. S., G. W. MOY, AND V. D. VACQUIER. 1983. Purification and characterization of sea urchin egg cortical granules. *J. Cell Biol.* **95:** 924–932.

KUEHL, F. A., AND R. W. EGAN. 1980. Prostaglandins, arachidonic acid, and inflammation. *Science* **210:** 978–984.

KUEHL, F. A., E. A. HAM, R. G. EGAN, H. W. DOUGHERTY, R. J. BONNEY, AND J. L. HUMES. 1982. Studies on a destructive oxidant released in the enzymatic reduction of prostaglandin G_2 and other hydroperoxy acids. Pp. 175–190 in *Pathology of Oxygen,* A. P. Autor, ed. Academic Press, NY.

KUNITZ, M. 1947. Crystalline soybean trypsin inhibitor. II. General properties. *J. Gen. Physiol.* **30:** 291–310.

LALLIER, R. 1971. Effects of various inhibitors of protein cross-linking on the formation of the fertilization membrane. *Experientia* **27:** 1323–1324.

LAPETINA, E. G., M. M. BILLAH, AND P. CUATRECASAS. 1981. The initial action of thrombin on platelets. Conversion of phosphatidylinositol to phosphatidic acid preceding the production of arachidonic acid. *J. Biol. Chem.* **256:** 5037–5040.

LAYCHOCK, S. G., AND J. W. PUTNEY. 1982. Roles of phospholipid metabolism in secretory cells. Pp. 53–105 in *Cellular Regulation of Secretion and Release,* P. M. Conn, ed. Academic Press, NY.

LEVINE, A. E., AND K. A. WALSH. 1979. Involvement of an acrosin-like enzyme in the acrosome reactin of sea urchin sperm. *Dev. Biol.* **72:** 126–137.

LILLIE, F. R. 1919. *Problems of Fertilization.* University of Chicago Press, Chicago, IL. 278 pp.

LONGO, F. J. 1973. An ultrastructural analysis of polyspermy in the surf clam *Spisula solidissima*. *J. Exp. Zool.* **183:** 153–180.

LONGO, F. J. 1977. An ultrastructural study of cross-fertilization (*Arbacia* ♀ × *Mytilus* ♂). *J. Cell Biol.* **73:** 14–26.

LONGO, F. J. 1978. Insemination of immature sea urchin (*Arbacia punctulata*) eggs. *Dev. Biol.* **62:** 271–291.

LONGO, F. J. 1980. Reinsemination of fertilized sea urchin (*Arbacia punctulata*) eggs. *Dev. Growth Diff.* **22:** 219–227.

LONGO, F. J. 1983. Cytoplasmic and sperm nuclear transformations in fertilized ammonia-activated sea urchin (*Arbacia punctulata*) eggs. *Gamete Res.* **8:** 65–78.

LONGO, F. J. 1984. Transformations of sperm nuclei incorporated into sea urchin (*Arbacia punctulata*) embryos at different stages of the cell cycle. *Dev. Biol.* **103:** 168–181.

LONGO, F. J., AND E. ANDERSON. 1970a. The effects of nicotine on fertilization in the sea urchin, *Arbacia punctulata*. *J. Cell Biol.* **46:** 308–325.

LONGO, F. J., AND E. ANDERSON. 1970b. A cytological study of the relation of the cortical reaction to subsequent events of fertilization in urethane-treated eggs of the sea urchin, *Arbacia punctulata*. *J. Cell Biol.* **47:** 646–665.

LONGO, F. J., AND H. SCHUEL. 1973. An ultrastructural examination of polyspermy induced by soybean trypsin inhibitor in the sea urchin *Arbacia punctulata*. *Dev. Biol.* **34:** 187–199.

LONGO, F. J., H. SCHUEL, AND W. L. WILSON. 1974. Mechanism of soybean trypsin inhibitor induced polyspermy as determined by an analysis of refertilized sea urchin (*Arbacia punctulata*) eggs. *Dev. Biol.* **41:** 193–201.

LONNING, S. 1967. Electron microscopic studies of the block to polyspermy. The influence of trypsin, soybean trypsin inhibitor and chloral hydrate. *Sarsia* **30:** 107–116.

LOPO, A. C. 1983. Sperm-egg interactions in invertebrates. Pp. 269–324 in *Mechanism and Control of Animal Fertilization,* J. F. Hartmann, ed. Academic Press, NY.

LUCY, J. A. 1970. The fusion of biological membranes. *Nature* **227:** 815–817.

LUNDBLAD, G. 1954. Proteolytic activity in sea urchin gametes. IV. Further investigations of proteolytic enzymes of the egg. *Ark. Kemi* **7:** 127–157.

LYNN, J. W., AND E. L. CHAMBERS. 1984. Voltage clamp studies of fertilization in sea urchin eggs. I. Effect of clamped membrane potential on sperm entry, activation, and development. *Dev. Biol.* **102:** 98–109.

MARGOLIASH, E., AND A. NOVOGRODSKY. 1960. Irreversible reaction of 3-amino-1,2,4-triazole and related inhibitors with the protein of catalase. *Biochem. J.* **74:** 339–348.

MAZIA, D. 1961. Mitosis and the physiology of cell division. Pp. 77–412 in *The Cell,* Vol. 3, J. Brachet and A. E. Mirsky, eds. Academic Press, NY.

MEIZEL, S., AND K. O. TURNER. 1983. The effects of products and inhibitors of arachidonic acid metabolism on an exocytotic event, the acrosome reaction. *J. Cell Biol.* **97:** 180a.

METZ, C. B. 1967. Gamete surface components and their role in fertilization. Pp. 163–236 in *Fertilization* Vol. 1, C. B. Metz and A. Monroy, eds. Academic Press, NY.

METZ, C. B., H. SCHUEL, AND E. R. BISCHOFF. 1964. Inhibition of the fertilizing capacity of sea urchin sperm by papain digested, non-agglutinating antibody. *J. Exp. Zool.* **155:** 261–272.

MONROY, A., AND F. ROSATI. 1983. A comparative analysis of sperm-egg interaction. *Gamete Res.* **7:** 85–102.

MORSE, D. E., H. DUNCAN, N. HOOKER, AND A. MORSE. 1977. Hydrogen peroxide induces spawning in mollusks, with activation of prostaglandin endoperoxide synthetase. *Science* **196:** 298–300.

MOSER, F. 1939. Studies on a cortical layer response to stimulating agents in the *Arbacia* egg. I. Response to insemination. *J. Exp. Zool.* **80:** 423–445.

MOSS, R., R. SCHUEL, AND H. SCHUEL. 1983. FPL-55712, a leukotriene antagonist, promotes polyspermy in sea urchins. *Biol. Bull.* **165:** 516.

NAKANO, E. 1956. Physiological studies of refertilization of the sea urchin egg. *Embryologia* **3:** 139–165.

NAKANO, E., A. HINO, AND K. FURUSE. 1984. Effects of surfactants on the fertilizing capacity and acrosome reaction of sea urchin spermatozoa. *Gamete Res.* **9:** 115–125.

NATHAN, C. 1982. Secretion of oxygen intermediates: role in effector functions of activated macrophages. *Fed. Proc.* **41:** 2206–2211.

NELSON, N. A., R. C. KELLY, AND R. A. JOHNSON. 1982. Prostaglandins and the arachidonic acid cascade. *Chem. Engin. News* **60:** 30–45.

NEURATH, H. 1984. Evolution of proteolytic enzymes. *Science* **224:** 350–357.

NEURATH, H., AND K. A. WALSH. 1976. Role of proteolytic enzymes in biological regulation (a review). *Proc. Natl. Acad. Sci.* **73:** 3825–3832.

NIMAN, H. L., B. R. HOUGH-EVANS, V. D. VACQUIER, R. J. BRITTEN, R. A. LERNER, AND E. H. DAVIDSON. 1984. Proteins of the sea urchin egg vitelline layer. *Dev. Biol.* **102:** 390–401.

NISHIOKA, D., AND N. CROSS. 1978. The role of external sodium in sea urchin fertilization. Pp. 403–413 in *Cell Reproduction in Honor of Daniel Mazia,* E. R. Dirkson, D. M. Prescott, and C. F. Fox, eds. Academic Press, NY.

NOMURA, T., AND H. OGATA. 1976. Distribution of prostaglandins in the animal kingdom. *Biochim. Biophys. Acta* **431:** 127–131.

NORTHRUP, J. H., AND R. G. HUSSEY. 1923. A method for the quantitative determination of trypsin and pepsin. *J. Gen. Physiol.* **5:** 353–358.

NUCCITELLI, R., AND R. D. GREY. 1984. Controversy over the fast, partial, temporary block to polyspermy in sea urchins: a reevaluation. *Dev. Biol.* **103:** 1–17.

OHMAN, L. 1945. On the lipids of the sea urchin egg. *Arkiv. Zool.* **36A:** 1–95.

PAREDES, J. M., AND S. J. WEISS. 1982. Human neutrophils transform prostaglandins by a myeloperoxidase-dependent mechanism. *J. Biol. Chem.* **257:** 2738–2740.

PAUL, M., AND R. N. JOHNSTON. 1978. Uptake of Ca^{2+} is one of the earliest responses to fertilization of sea urchin eggs. *J. Exp. Zool.* **203:** 143–149.

PAYAN, P., J. GIRARD, R. CHRISTEN, AND C. SARDET. 1981. Na^+ movements and their oscillations during fertilization and the cell cycle in sea urchin eggs. *Exp. Cell Res.* **134:** 339–344.

PERRY, G. 1979. Studies on the calcium-stimulated oxidations of the sea urchin egg. Ph.D. Thesis, University of California, San Diego.

PERRY, G., AND D. EPEL. 1981. Ca^{2+}-stimulated production of H_2O_2 from naphthoquinone oxidation in *Arbacia* eggs. *Exp. Cell Res.* **134:** 65–72.

POSTE, G., AND A. C. ALLISON. 1973. Membrane fusion. *Biochem. Biophys. Acta* **300:** 421–467.

PRESLEY, R., AND P. F. BAKER. 1970. Kinetics of fertilization in the sea urchin: a comparison of methods. *J. Exp. Biol.* **52:** 455–468.

RACOWSKY, C., AND J. D. BIGGERS. 1983. Are blastocyst prostaglandins produced endogenously? *Biol. Reprod.* **29:** 379–388.

REESE, E. S. 1966. The complex behavior of echinoderms. Pp. 157–218 in *Physiology of Echinodermata,* R. A. Boolootian, ed. J. Wiley and Sons, NY.

RIBOT, H., S. J. DECKER, AND W. H. KINSEY. 1983. Preparation of plasma membranes from fertilized sea urchin eggs. *Dev. Biol.* **97:** 494–499.

ROSSIGNOL, D. P., A. J. ROSCHELLE, AND W. J. LENNARZ. 1981. Sperm-egg binding: identification of a species-specific sperm receptor from eggs of *Strongylocentrotus purpuratus. J. Supramol. Struct. Cell Biochem.* **15:** 347–358.

ROTHSCHILD, L. 1950. The physiology of sea urchin spermatozoa: catalase. *J. Exp. Biol.* **26:** 396–409.

ROTHSCHILD, L. 1953. The fertilization reaction in the sea urchin. The induction of polyspermy by nicotine. *J. Exp. Biol.* **30:** 57–67.

ROTHSCHILD, L. 1956. *Fertilization.* Methuen and Co., London. 170 pp.

ROTHSCHILD, L., AND M. M. SWANN. 1950. The fertilization reaction of the sea-urchin egg. The effect of nicotine. *J. Exp. Biol.* **27:** 400–406.

ROTHSCHILD, L., AND M. M. SWANN. 1952. The fertilization reaction in the sea urchin. The block to polyspermy. *J. Exp. Biol.* **29:** 469–483.

RUBIN, R. P. 1982. Calcium-phospholipid interactions in secretory cells: a new perspective on stimulus-secretion coupling. *Fed. Proc.* **41:** 2181–2187.

RUNNSTROM, J. 1966. The vitelline membrane and cortical particles in sea urchin eggs and their function in maturation and fertilization. *Adv. Morphogen.* **5:** 221–325.

RUNNSTROM, J., B. E. HAGSTROM, AND P. PERLMAN. 1959. Fertilization. Pp. 327–397 in *The Cell* Vol. I, J. Brachet and A. E. Mirsky, eds. Academic Press, NY.

SAMUELSSON, B. 1983. Leukotrienes: mediators of immediate hypersensitivity reactions and inflammation. *Science* **220:** 568–575.

SANO, K., AND H. KANATANI. 1980. External calcium ions are requisite for fertilization of sea urchin eggs by spermatozoa with reacted acrosomes. *Dev. Biol.* **78:** 242–246.

SARDET, C., D. CARRE, M. P. COSSON, J. COSSON, P. CHANG, P. PAYAN, AND J. P. GIRARD. 1982. Some aspects of fertilization in marine invertebrates. Pp. 185–210 in *Membranes in Growth and Development,* J. F. Hoffman, G. H. Giebisch, and L. Bolis, eds. Alan R. Liss, Inc., NY.

SAWADA, H., M. MIURA, H. YOKOSAWA, AND S. ISHII. 1984. Purification and characterization of trypsin-like enzyme from sea urchin eggs: substrate specificity and physiological role. *Biochem. Biophys. Res. Comm.* **121:** 598–604.

SCHATTEN, G. 1978. The block to polyspermy in the sea urchin. Pp. 391–402 in *Cell Reproduction in Honor of Daniel Mazia,* E. R. Dirksen, D. M. Prescott, and C. F. Fox, eds. Academic Press, NY.

SCHMELL, E. D., AND B. J. GULYAS. 1980. Ovoperoxidase activity in ionophore treated mouse eggs. II. Evidence for the enzyme's role in hardening the zona pellucida. *Gamete Res.* **3:** 279–290.

SCHMELL, E., AND W. J. LENNARZ. 1974. Phospholipid metabolism in eggs and embryos of the sea urchin, *Arbacia punctulata. Biochemistry* **13:** 4114–4121.

SCHMELL, E., B. J. EARLES, C. BREAUX, AND W. J. LENNARZ. 1977. Identification of a sperm receptor on the surface of eggs of the sea urchin *A. punctulata. J. Cell Biol.* **72:** 35–46.

SCHMELL, E. D., B. J. GULYAS, AND J. L. HEDRICK. 1983. Egg surface changes during fertilization and the molecular mechanism of the block to polyspermy. Pp. 365–413 in *Mechanism and Control of Animal Fertilization,* J. F. Hartmann, ed. Academic Press, NY.

SCHMIDT, T., C. PATTON, AND D. EPEL. 1982. Is there a role for the Ca^{2+} influx during fertilization of the sea urchin egg? *Dev. Biol.* **90:** 284–290.

SCHUEL, H. 1978. Secretory functions of egg cortical granules in fertilization and development: a critical review. *Gamete Res.* **1:** 299–382.

SCHUEL, H. 1984. Functions of egg cortical granules. In *Biology of Fertilization* Vol. 2, C. B. Metz and A. Monroy, eds. Academic Press, NY. (In press.)

SCHUEL, H., AND R. SCHUEL. 1981. A rapid sodium-dependent block to polyspermy in sea urchin eggs. *Dev. Biol.* **87:** 249–258.

SCHUEL, H., W. L. WILSON, K. CHEN, AND L. LORAND. 1973. A trypsin-like proteinase localized in cortical granules isolated from sea urchin eggs by zonal centrifugation. Role of the enzyme in fertilization. *Dev. Biol.* **34:** 175–186.

SCHUEL, H., J. W. KELLY, E. R. BERGER, AND W. L. WILSON. 1974. Sulfated acid mucopolysaccharides in the cortical granules of eggs. Effects of quaternary ammonium salts on fertilization. *Exp. Cell Res.* **88:** 24–30.

SCHUEL, H., F. J. LONGO, W. L. WILSON, AND W. TROLL. 1976a. Polyspermic fertilization of sea urchin eggs treated with protease inhibitors: localization of sperm receptor sites at the egg surface. *Dev. Biol.* **49:** 178–184.

SCHUEL, H., W. TROLL, AND L. LORAND. 1976b. Physiological responses of sea urchin eggs to stimulation by calcium ionophore A23187 analysed with protease inhibitors. *Exp. Cell Res.* **103:** 442–447.

SCHUEL, H., P. V. DANDEKAR, AND R. SCHUEL. 1982a. Urea parthenogenetically activates the cortical reaction and elongation of microvilli in eggs of the sea urchin, *Strongylocentrotus purpuratus. Biol. Bull.* **163:** 337–347.

SCHUEL, H., R. SCHUEL, P. DANDEKAR, J. BOLDT, AND R. G. SUMMERS. 1982b. Sodium requirements in hardening of the fertilization envelope and embryonic development in sea urchins. *Biol. Bull.* **162:** 202–213.

SCHUEL, H., E. TRAEGER, R. SCHUEL, AND J. BOLDT. 1983. Evidence for an arachidonic acid cascade mediated block to polyspermy in sea urchins. *J. Cell Biol.* **97:** 28a.

SCHUEL, H., E. TRAEGER, R. SCHUEL, AND J. BOLDT. 1984. Anti-inflammatory drugs promote polyspermic fertilization in sea urchins. *Gamete Res.* **10:** 9–19.

SEGALL, G. K., AND W. J. LENNARZ. 1979. Chemical characterization of the component of the jelly coat from sea urchin eggs responsible for induction of the acrosome reaction. *Dev. Biol.* **71:** 33–48.

SEGALL, G. K., AND W. J. LENNARZ. 1981. Jelly coat induction of the acrosome reaction in echinoid sperm. *Dev. Biol.* **86:** 87–93.

SHAPIRO, B. M. 1975. Limited proteolysis of some egg components is an early event following fertilization of the sea urchin, *Strongylocentrotus purpuratus*. *Dev. Biol.* **46:** 88–102.

SHAPIRO, B. M., AND E. M. EDDY. 1980. When sperm meets egg: biochemical mechanisms of gamete interaction. *Int. Rev. Cytol.* **66:** 257–302.

SHEARD, P., M. C. HOLROYDE, A. M. GHELANI, J. R. BANTICK, AND T. B. LEE. 1982. Antagonists of SRS-A and leukotrienes. Pp. 229–235 in *Leukotrienes and Other Lipoxygenase Products,* B. Samuelsson and R. Paoletti, eds. Raven Press, NY.

SHEN, S. S. 1983. Membrane properties and intracellular ion activities of marine invertebrate eggs and their changes during activation. Pp. 213–267 in *Mechanism and Control of Animal Fertilization,* J. F. Hartmann, ed. Academic Press, NY.

SHEN, S. S., AND R. A. STEINHARDT. 1984. Time and voltage windows for reversing the electrical block to fertilization. *Proc. Natl. Acad. Sci.* **81:** 1436–1439.

SHIMADA, H., H. TERAYAMA, A. FUJIWARA, AND I. YASUMASU. 1982. Melittin, a component of bee venom activates unfertilized sea urchin eggs. *Dev. Growth Differ.* **24:** 7–16.

SMITH, D. C., AND S. J. KLEBANOFF. 1970. A uterine fluid-mediated sperm-inhibitory system. *Biol. Reprod.* **3:** 229–235.

STEINHARDT, R. A., AND D. EPEL. 1974. Activation of sea urchin eggs by calcium ionophore. *Proc. Natl. Acad. Sci.* **71:** 1915–1919.

STEINHARDT, R. A., L. LUNDIN, AND D. MAZIA. 1971. Bioelectric responses of the echinoderm egg to fertilization. *Proc. Natl. Acad. Sci.* **68:** 2426–2430.

SUGIYAMA, M. 1951. Re-fertilization of the fertilized eggs of the sea urchin. *Biol. Bull.* **101:** 335–344.

SUMMERS, R. G., AND B. L. HYLANDER. 1976. Primary gamete binding. Quantitative determination of its specificity in echinoid fertilization. *Exp. Cell Res.* **100:** 190–194.

SUMMERS, R. G., B. L. HYLANDER, L. H. COLWIN, AND A. L. COLWIN. 1975. The functional anatomy of the echinoderm spermatozoan and its interaction with the egg at fertilization. *Am. Zool.* **15:** 523–551.

TAGLIETTI, V. 1979. Early electrical responses to fertilization in sea urchin eggs. *Exp. Cell Res.* **120:** 448–451.

THAKKAR, J. K., J. EAST, AND R. C. FRANSON. 1984. Modulation of phospholipase A_2 activity associated with human sperm membranes by divalent cations and calcium antagonists. *Biol. Reprod.* **30:** 679–686.

TROLL, W., G. WITZ, B. GOLDSTEIN, D. STONE, AND T. SUGIMURA. 1982. The role of free oxygen radicals in tumor promotion and carcinogenesis. *Carcinogenesis* **7:** 593–597.

TURNER, P. R., L. A. JAFFE, AND M. P. SHEETZ. 1983. Polyphosphoinositide changes post fertilization in sea urchin eggs. *J. Cell Biol.* **97:** 29a.

TYLER, A., AND B. S. TYLER. 1966a. The gametes; some properties and procedures. Pp. 639–682 in *Physiology of Echinodermata,* R. A. Boolootian, ed. J. Wiley and Sons, NY.

TYLER, A., AND B. S. TYLER. 1966b. Physiology of fertilization and early development. Pp. 683–741 in *Physiology of Echinodermata,* R. A. Boolootian, ed. J. Wiley and Sons, NY.

TYLER, A., A. MONROY, C. Y. KAO, AND H. GRUNDFEST. 1956a. Membrane potential and resistance of the starfish egg before and after fertilization. *Biol. Bull.* **111:** 153–177.

TYLER, A., A. MONROY, AND C. B. METZ. 1956b. Fertilization of fertilized sea-urchin eggs. *Biol. Bull.* **110:** 184–195.

VACQUIER, V. D. 1979. The fertilizing capacity of sea urchin sperm rapidly decreases after induction of the acrosome reaction. *Dev. Growth Differ.* **21:** 61–69.

VACQUIER, V. D., AND J. E. PAYNE. 1973. Methods for quantitating sea urchin sperm-egg binding. *Exp. Cell Res.* **82:** 227–235.

VACQUIER, V. D., D. EPEL, AND L. A. DOUGLAS. 1972a. Sea urchin eggs release protease activity at fertilization. *Nature* **240:** 34–36.

VACQUIER, V. D., M. J. TEGNER, AND D. EPEL. 1972b. Protease activity establishes the block against polyspermy in sea urchin eggs. *Nature* **240:** 352–353.

VACQUIER, V. D., M. J. TEGNER, AND D. EPEL. 1973. Protease released from sea urchin eggs at fertilization alters the vitelline layer and aids in preventing polyspermy. *Exp. Cell Res.* **80:** 111–119.

VACQUIER, V. D., B. BRANDRIFF, AND C. G. GLABLE. 1979. The effect of soluble egg jelly on the fertilizability of acid-dejellied sea urchin eggs. *Dev. Growth Differ.* **21:** 47–60.

VERON, M., C. FOERDER, E. M. EDDY, AND B. M. SHAPIRO. 1977. Sequential biochemical and morphological events during assembly of the fertilization membrane of the sea urchin. *Cell* **10:** 321–328.

VOGT, W. 1978. Role of phospholipase A_2 in prostaglandin formation. *Adv. Prosta. Throm. Res.* **3:** 89–95.

WEISSMANN, G., C. SERHAN, J. E. SMOLEN, H. M. KORCHAK, R. FRIEDMAN, AND H. P. KAPLAN. 1982. Stimulus-secretion coupling in the human neutrophil: the role of phosphatidic acid and oxidized fatty acids in the translocation of calcium. Pp. 259–272 in *Leukotrienes and Other Lipoxygenase Products,* B. Samuelsson, and R. Paoletti, eds. Raven Press, NY.

WHITAKER, M. J., AND R. A. STEINHARDT. 1982. Ionic regulation of egg activation. *Q. Rev. Biophys.* **15:** 593–666.

WHITAKER, M. J., AND R. A. STEINHARDT. 1983. Evidence in support of the hypothesis of an electrically mediated fast block to polyspermy in sea urchin eggs. *Dev. Biol.* **95:** 244–248.

WILSON, E. B. 1900. *The Cell in Development and Inheritance,* 2nd Ed., MacMillan, London. 483 pp.

WOLF, D. P. 1981. The mammalian egg's block to polyspermy. Pp. 183–197 in *Fertilization and Embryonic Development in Vitro,* L. Mastroianni, and J. D. Biggers, eds. Plenum Publ. Corp., NY.

YAMADA, Y., AND K. AKETA. 1981. Vitelline layer lytic activity in sperm extracts of sea urchin, *Hemicentrotus pulcherrimus. Gamete Res.* **4:** 193–202.

YOSHIDA, M., AND K. AKETA. 1978. Localization of species-specific sperm-binding factor in sea urchin eggs with immunofluorescent probe. *Acta Embryol. Exp.* **3:** 269–278.

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FINE STRUCTURE AND VITAL STAINING OF OSPHRADIUM OF THE SOUTHERN OYSTER DRILL, *THAIS HAEMASTOMA CANALICULATA* (GRAY) (PROSOBRANCHIA: MURICIDAE)

DAVID W. GARTON[1], RICHARD A. ROLLER, AND JOHN CAPRIO

Department of Zoology and Physiology, Louisiana State University, Baton Rouge, Louisiana 70803

ABSTRACT

The morphology of the osphradium of *Thais haemastoma canaliculata* (Gray) was examined using light microscopy, SEM, and TEM. The osphradium is composed of approximately 150–200 lamellae, each of which is divided into two distinct regions by a groove situated parallel to the dorsal edge of the organ. The dorsal one-fourth of each lamella is covered by dense cilia that are assumed to generate water currents about the osphradium. Ciliary tufts, located in small depressions, and numerous secretory cells are distributed uniformly on the ventral three-fourths of the lamellae. A thin tract of cilia borders the ventral edge of each lamella. The overall cellular organization is less complex than has been reported previously in other marine prosobranchs. Selective staining of putative chemoreceptors was performed using Procion Brilliant Yellow. Individual cells in the ventral region and the ventral edge of each lamella were Procion-positive. Results of this study suggest that ventral interlamellar regions and the ventral edge of each lamella are chemosensory regions, while the dorsal portion of each lamella is indifferent epithelium.

INTRODUCTION

The chemosensory function of the osphradium in prosobranch gastropods is well established (Brown and Noble, 1960; Bailey and Laverack, 1966; Bailey and Benjamin, 1968). Ultrastructural studies on several prosobranch species reveal a similar overall pattern of cellular organization, although there is little agreement on the functional interpretation of individual cell types (Welsch and Storch, 1969; Alexander, 1970; Crisp, 1973; Alexander and Weldon, 1975; Newell and Brown, 1977). Five different types of presumed sensory cells ("Sinneszelle") were identified from transmission electron microscopy of the osphradium of *Buccinium undatum* (Welsch and Storch, 1969). These five cell types, dubbed Si1-Si5 (Crisp, 1973), were thought to be either chemoreceptors or proprioreceptors. Crisp (1973) found cell types Si1-Si4 in the osphradia of five prosobranch species, but she concluded that cell types Si1 and Si2 were not sensory receptors because neither axons nor intracellular vesicles were observed. The presumed sensory receptors, Si3 (similar to a free nerve ending) and Si4 (similar to a primary receptor) were located primarily in a specialized region of the interlamellar surface (Crisp, 1973). Regional specialization of lamellar epithelium has been observed in other marine prosobranchs (Alexander and Weldon, 1975; Newell and Brown, 1977). Newell and Brown (1977), on the basis of "presynaptic vesicles," reported that some ciliated cells in the osphradium of *Bullia digitalis* might be secondary receptors. As yet ultrastructural observations have not elucidated the primary or secondary nature of receptors and no electrophysiological data have confirmed the conclusions drawn from these ultrastructural observations.

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[1] Present address: Department of Ecology and Evolution, State University of New York at Stony Brook, Stony Brook, New York 11794.

The present investigation was initiated to study the gross morphology and ultrastructure of the osphradial epithelium of *Thais haemastoma canaliculata* (Gray) using light microscopy, SEM, and TEM and to identify putative chemoreceptor cells by vital staining with Procion Brilliant Yellow.

Materials and Methods

Adult oyster drills, *Thais haemastoma canaliculata* (Gray), were collected from the vicinity of Caminada Pass near Grand Isle, Louisiana. Drills were transported to the laboratory and placed in a 38 l aquarium at room temperature (23–25°C) and 20 ‰S (Instant Ocean® Seawater Mix).

Snails were removed from their shells by gently cracking open the shell at the base of the body whorl and severing the columellar muscle. The dorsal mantle was dissected open and folded back to expose the osphradium at the base of the ctenidium. The osphradium was dissected from the ctenidium and preserved for histological examination.

For scanning electron microscopy (SEM), osphradia were fixed overnight with 2.5% gluteraldehyde in 0.2 *M* sodium cacodylate-sucrose buffer (585 mOsm; pH = 8.0). Specimens were rinsed in distilled water to remove all buffer salts, dehydrated in acidified 2,2-dimethoxypropane, critical-point dried in CO_2 and coated with 200Å of Au/Pd. Osphradia were examined at 25kV with a Hitachi S-500 SEM.

For transmission electron microscopy (TEM), osphradia were fixed overnight with 2.5% gluteraldehyde in 0.2 *M* sodium cacodylate-sucrose buffer (585 mOsm; pH = 8.0) and post-fixed for 2 h in 1% OsO_4 buffered in 0.2 *M* sodium cacodylate (pH = 8.0). Specimens were dehydrated in ethanol and embedded in Epon. Ultrathin sections were placed on copper grids and contrasted with uranyl acetate-lead citrate. Sections were examined with a JEOL 100-CX TEM at 80 kV.

Vital staining of epithelial receptor cells in the osphradium was accomplished by a modification of the method of Holl (1981). Live drills acclimated to 7.5 ‰S were anesthetized by chilling on ice. The shell of each snail was gently cracked at the body whorl, immediately over the osphradium. The mantle edge was cut and reflected and the exposed osphradium was periodically irrigated for 30 minutes with a 4% solution of Procion Brilliant Yellow in 0.1 *M* KCl. For four species of freshwater fish, Holl (1981) used a 0.01 *M* KCl staining solution, a concentration we considered too low for an estuarine gastropod. *Thais haemastoma canaliculata* can be readily acclimated to low salinity (Garton and Stickle, 1980), therefore a 0.1 *M* KCl solution (200 mOsm/kg) was used so that osmotic differences between the staining solution and the hemolymph would be minimized in low salinity acclimated snails (7.5 ‰S equals 223 mOsm/kg). Excess stain was flushed away with 7.5 ‰S sea water. The osphradium was post incubated at room temperature (24°C) for 30 minutes, removed from the snail and fixed in buffered formalin (pH = 6.5). The specimen was dehydrated in alcohol, cleared in xylene and embedded in paraffin. Sections, 7 μm, were mounted on slides and the paraffin removed with xylene. Sections were coated with immersion oil and viewed at 390–490 nm (mirror—510 nm; suppression—515 nm) with an epifluorescence light microscope equipped with a Leitz H2 filter block. Non-stained osphradia were examined as controls.

Results

SEM and TEM observations

The general morphology of the osphradium in *Thais haemastoma canaliculata* is typical of prosobranch gastropods, being bipectinate and containing approximately

150–200 lamellae (Fig. 1). The pseudostratified epithelium is separated from the central region of the lamella by a distinct basal lamina. Connective tissue, muscle fibers, nerves, and blood spaces are present in the central region of each lamella (Fig. 2). In the live snail the lamellae are active and capable of independent movement.

In *Thais haemastoma canaliculata* a groove 10–15 μm wide and 5 μm deep runs parallel to the dorsal edge of each lamellae and separates the lamellar epithelium into two distinct regions (Figs. 3, 4). A thin tract of cilia is present along the ventro-lateral edge of each lamella (Figs. 3, 5). The epithelium dorsal to the groove is composed entirely of a single layer of ciliated cells. There are relatively few mitochondria in these cells, and the mitochondrial cristae are diffuse, indicating a relatively low metabolic rate (Munn, 1974). Ciliary rootlets are short and many microvilli cover the cell surface. The epithelium ventral to the lamellar groove, occupying approximately

FIGURE 1. SEM of the anterior portion of the osphradium (Os) showing its relation to the ctenidium (Ct) and incurrent siphon (S). Me–mantle epithelium; R–raphe.

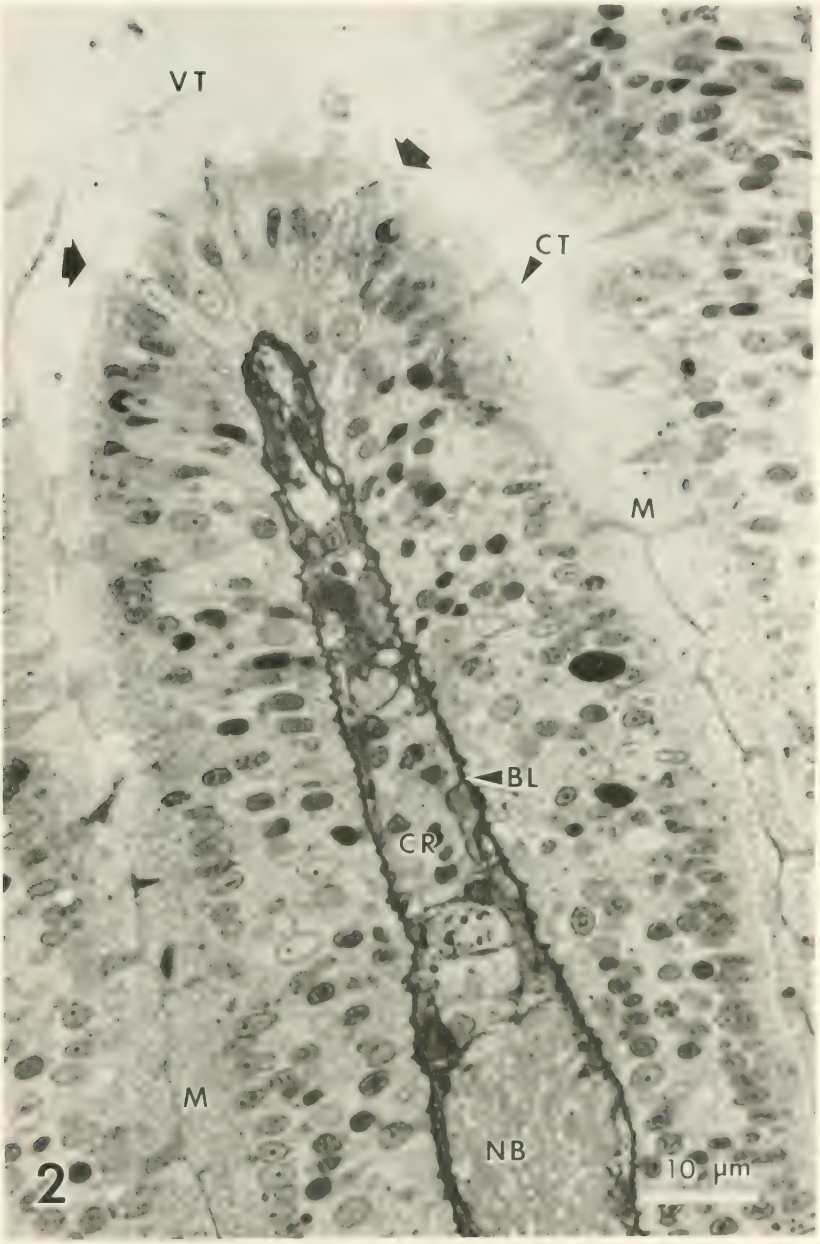


FIGURE 2. Longitudinal thin section (1 μm) of a single osphradial lamella stained with Paragon. BL–basal lamina; CT–ciliary tuft; CR–central region; M–mucus layer; NB–nerve bundle; VT–ventral ciliary tract (between arrows).

three-fourths of the surface area of each lamella, contains uniformly distributed, tufted ciliated cells (Figs. 6, 7). These cilia originate from cells lying depressed between neighboring cells. Numerous mitochondria with well developed cristae are concentrated

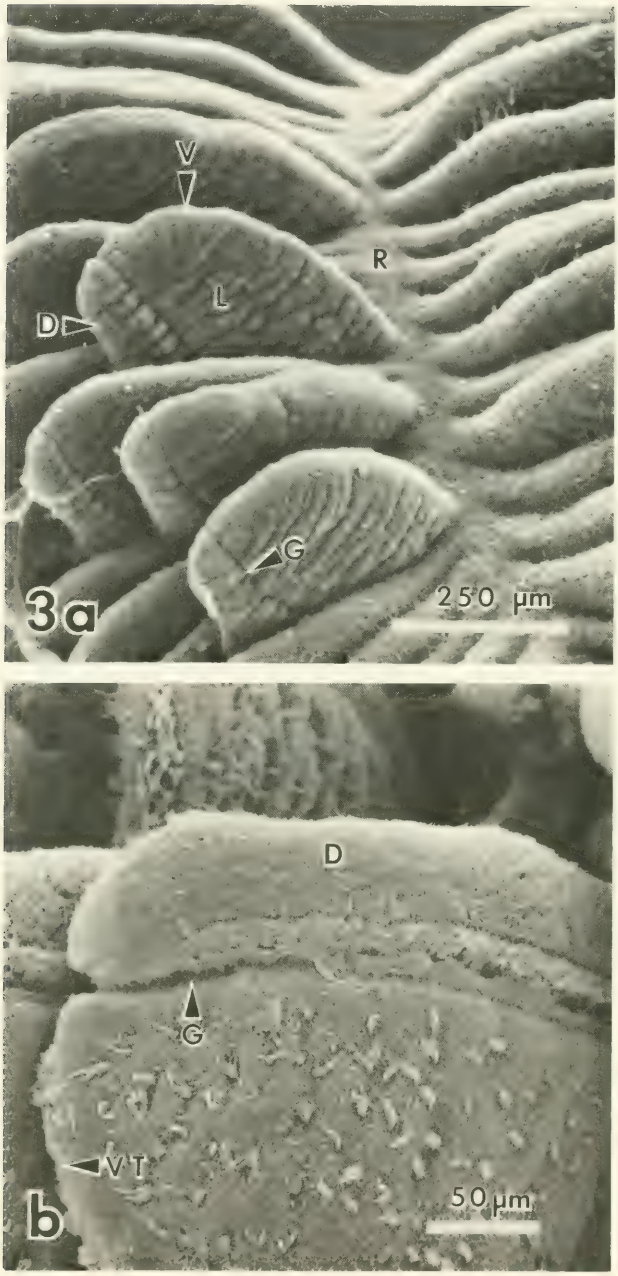
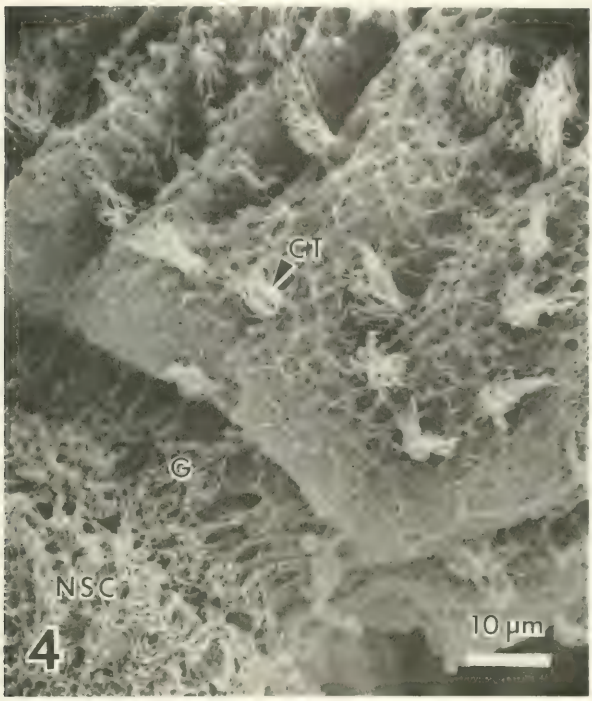


FIGURE 3a. SEM of an inverted osphradium showing directional references for the lamella. Note the unequal extension of individual lamellae. D–dorsal edge of lamella; G–lamellar groove; L–lamella; R–raphe; V–ventral edge of lamella. b. Lateral view of single lamella (SEM), orientation reversed from a. D–dorsal edge; G–lamellar groove; VT–ventral tract of cilia.

at the apical end of the ciliated cell (Fig. 8). Long ciliary rootlets extend basally through the aggregate of mitochondria. Ciliary tufts project laterally into the interlamellar space. The interlamellar space is filled with mucus and membrane-bound



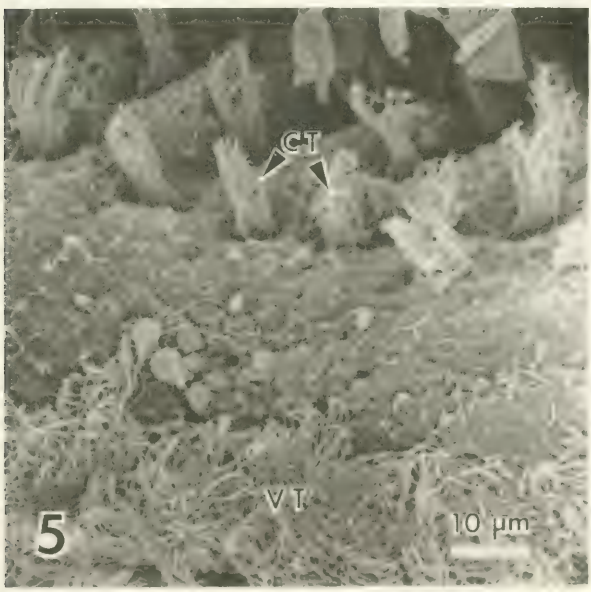


FIGURE 4. Transitional region on the dorsal edge of a single lamella (SEM). CT–cilia tuft; G–lamellar groove; NSC–non-sensory cilia.

FIGURE 5. Transitional region (SEM) on the ventral edge of a single lamella. CT–cilia tuft; VT–ventral tract of cilia.

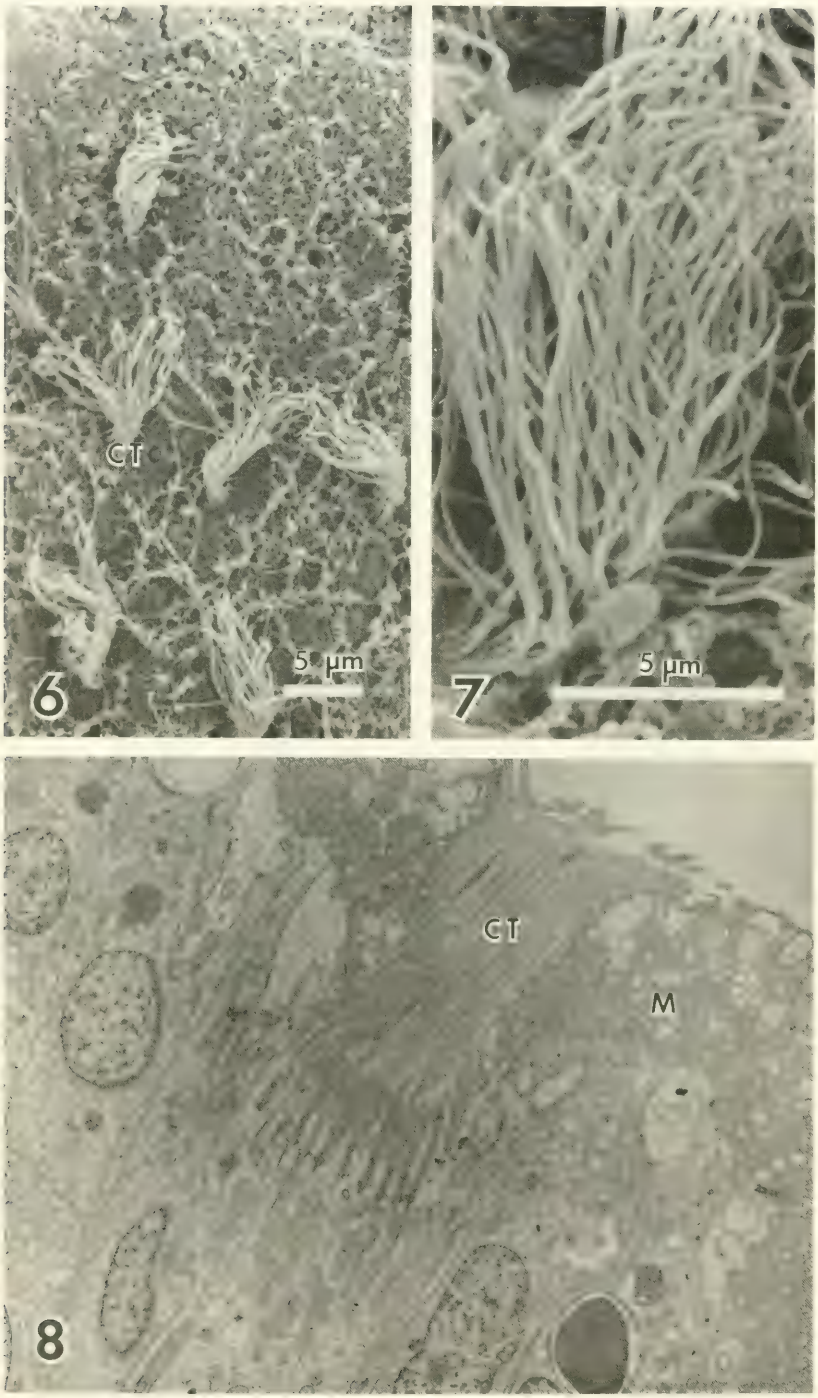


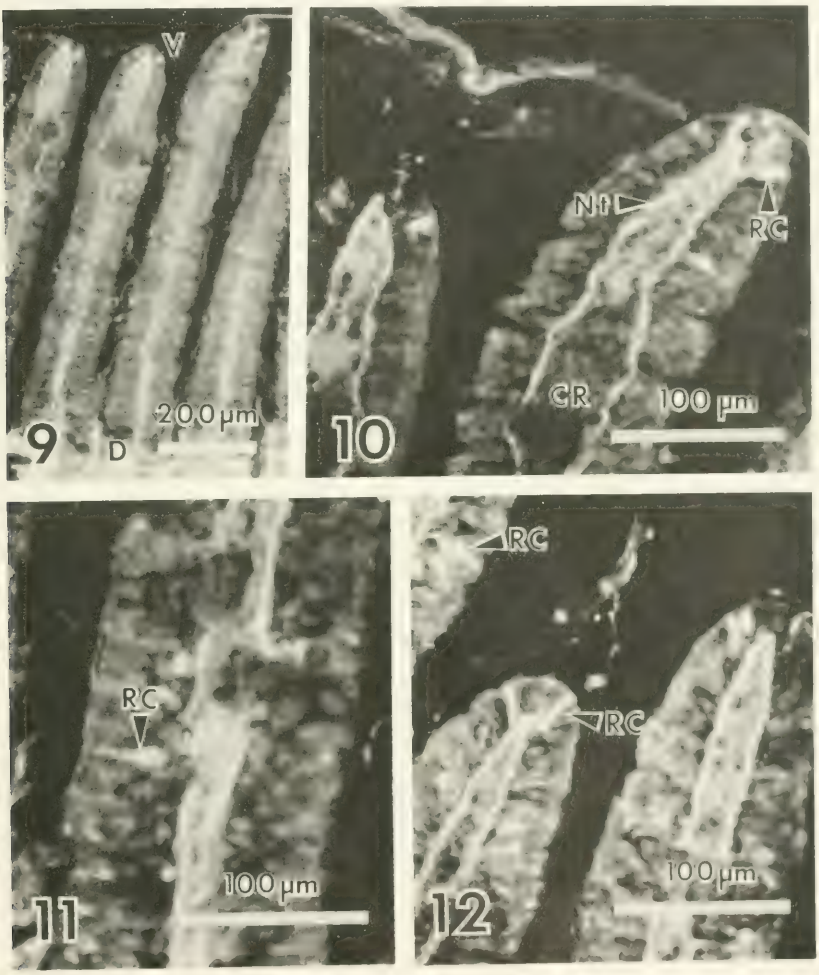
FIGURE 6. SEM of ciliary tufts (CT) on the interlamellar surface.
FIGURE 7. SEM of individual ciliary tuft in Figure 6.
FIGURE 8. TEM of ciliary tuft (CT) projecting into mucus layer (M). Magnification = 4125×.

secretory vesicles produced by the surrounding non-ciliated cells present in the ventral region of the lamellae (Fig. 8).

All cilia observed by TEM, regardless of location, were of the $9 \times 2 + 2$ fibril arrangement. Cilia were traced distally in many sections and no deviation from the $9 \times 2 + 2$ arrangement was observed. Nerve tracts were located in the central region of the lamellae but no axons or synapses bridging the basal lamina were observed.

Vital staining

Selective staining of individual receptor cells is shown in Figures 9–12. Epithelial cells present in the region ventral to the groove and along the ventro-lateral tract of



FIGURES 9–12. Fluorescent light micrographs of Procion Brilliant Yellow stained material. FIGURE 9. Low magnification of osphradial leaflets. D dorsal; V ventral. FIGURE 10. Procion-positive cells (RC) at ventral tip of lamella. CR–central region; Nt–nerve tract. FIGURE 11. Procion-positive cell (RC) in interlamellar region. FIGURE 12. Example of a Procion-positive cell (RC) at ventral or distal region of lamella.

cilia stained as well as nerve tracts in the central region (Figs. 9–12). Although the individual cells that were stained by Procion Brilliant Yellow could not be identified as specific cell types observed in the SEM and TEM material, their location suggests that tufted ciliated cells along the interlamellar surface and ciliated cells of the ventro-lateral tract were stained.

Discussion

The osphradial lamella in *Thais haemastoma canaliculata* (Gray) is divided into two morphologically distinct regions. This division has been observed in other prosobranchs (Crisp, 1973; Newell and Brown, 1977; Altner and Prillinger, 1980). Welsch and Storch (1969) and Crisp (1973) reported that the presumed sensory receptors are concentrated in the epithelium adjacent to the "transition zone" or groove on the lamella. In *Conus flavidus* the arrangement is more complex. Alexander (1970) and Alexander and Weldon (1975) observed presumptive receptor cells along interdigiform grooves on the interlamellar surfaces of the osphradial lamellae in *Conus flavidus.* These cells were also located in small depressions, similar to those in *T. haemastoma canaliculata.* Hackney *et al.* (1983) identified tufted, ciliated cells as putative chemoreceptors on the pallial tentacles of the limpet *Patella vulgata.* The gross morphology of these tufted cells is similar to that of the tufted ciliated cells observed in this study. Our observations imply that rather than being restricted to a specialized "transition zone," chemosensory cells are distributed over a major part of the osphradial lamella in *T. haemastoma canaliculata.*

Results of the vital staining in *Thais haemastoma canaliculata* also indicate that sensory cells (possibly chemosensory) are uniformly distributed across the interlamellar surface and ventro-lateral edge of the lamella. Procion-positive cells are denser along the ventral edge than across the interlamellar surface. Ventral edges of lamellae project into the inhalant water stream (the osphradium is located on the dorsal wall of the mantle cavity adjacent to the ctenidium), providing maximal exposure to environmental stimuli. After contacting ventral edges, water currents pass through mucus-filled interlamellar spaces, then along the dorsal edge of lamellae, adjacent to the mantle epithelium. Any chemosensory cells present in the dorsal region would come into contact with odorant molecules last.

Deviations from the $9 \times 2 + 2$ fibril arrangement in osphradial cilia have been observed in prosobranchs (Crisp, 1973). Crisp (1973) reported $(8 + 1)$, $(8 + 0)$, $(7 + 1)$, $(7 + 0)$, and $(6 + 1)$ arrangements. Other ciliary modifications for a putative chemoreceptor cell have been observed in an opistobranch (Davis and Matera, 1982; Matera and Davis, 1982). "Discocilia" were observed only in chemosensitive regions of the body of *Pleurobranchaea californica* (Davis and Matera, 1982). SEM and TEM observations in our study revealed no specialized modifications of cilia in different regions of the osphradial lamellae that might suggest chemosensory function. All cilia observed were of the $9 \times 2 + 2$ fibril arrangement.

Both primary and secondary receptors have been reported in osphradial receptors (Crisp, 1973; Newell and Brown, 1977). Crisp (1973) speculated that the Si3 and Si4 cell types are primary receptors, although no axons were observed. Given the volume of epithelial cells compared to axon sizes (tenths of microns), the probability that we could observe axonal connections in TEM material is quite low. Crisp (1973) reported no evidence for secondary receptors (synaptic vesicles); however, Newell and Brown (1977) reported chemical synapses in the osphradial epithelium of *Bullia* associated with tufted ciliated cells, although no high magnification figure of the vesicles was provided. In invertebrates, the great majority of sensory cells are primary receptors;

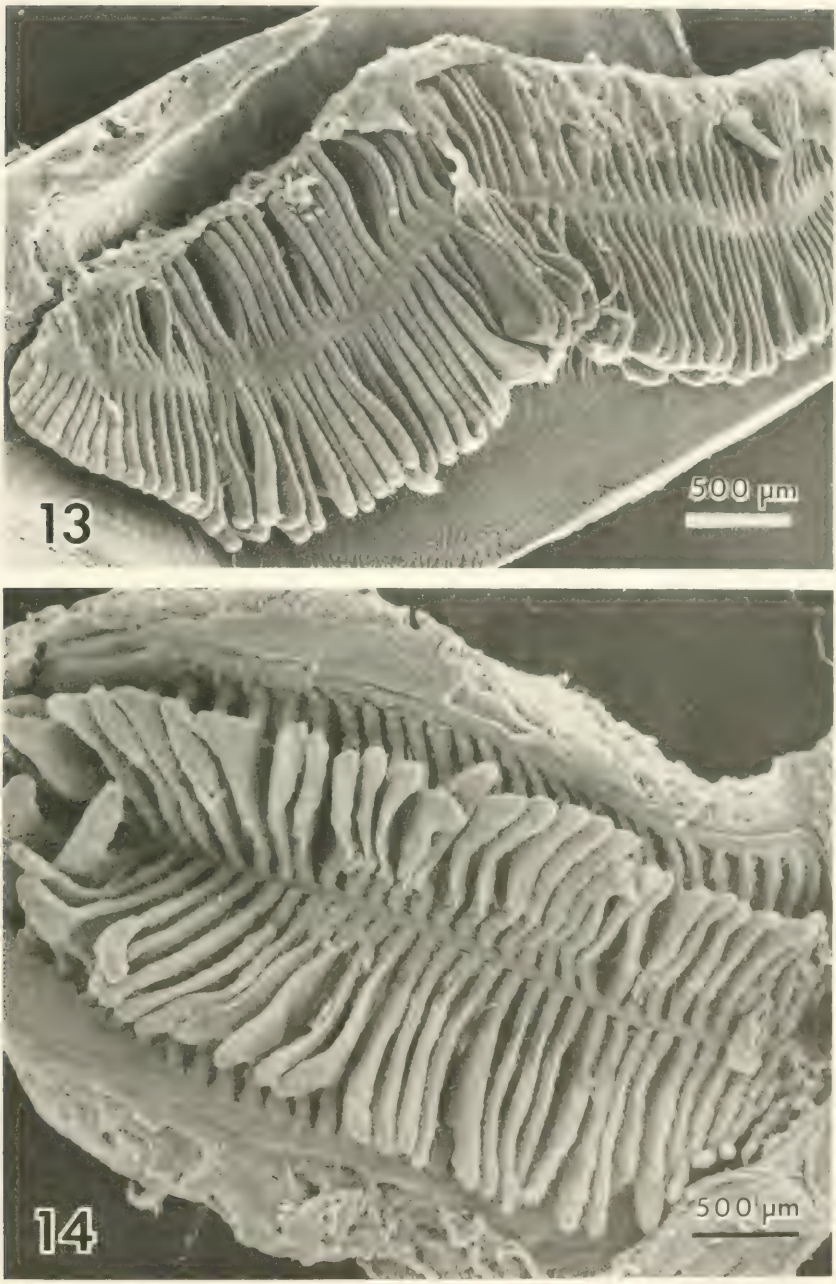


FIGURE 13. Scanning electron micrograph (SEM) of the ventral surface of the osphradium of the southern oyster drill, *Thais haemastoma canaliculata* (Gray). The osphradium is suspended from the roof of the mantle cavity and the lamellae project ventrally into the incurrent water stream. Anterior is to the left.

FIGURE 14. SEM of the dorsal surface of the olfactory rosette of the channel catfish, *Ictalurus punctatus*. The rosette is attached to the floor of the olfactory capsule and the lamellae project dorsally into the olfactory chamber. Anterior is to the right.

development of secondary receptors is considered a vertebrate characteristic (Bullock and Horridge, 1965). Nerve tracts associated with putative (chemo?)-receptors were observed in the Procion Brilliant Yellow stained material, although none were observed in TEM or fractured SEM specimens. Transport of the stain along the nerve tracts suggests that the selectively stained epithelial cells are primary receptor cells. Also, no synapses or intracellular vesicles indicative of secondary receptors were observed.

Cellular organization of the osphradial epithelium in *Thais haemastoma canaliculata* is less complex than has been reported for other prosobranchs (Crisp, 1973; Newell and Brown, 1977; Altner and Prillinger, 1980). The results of this study suggest a functional separation of different epithelial regions of each lamella (sensory *versus* non-sensory) similar to that found in many teleosts (Yamamoto, 1982). The gross morphology of the prosobranch osphradium (Fig. 13) and the teleost olfactory organ (Fig. 14) are strikingly similar, suggesting convergent evolution. Both organs are composed of numerous, laterally radiating lamellae organized into specialized sensory and indifferent epithelial regions (Caprio and Raderman-Little, 1978; Yamamato, 1982).

Because definitive axonal connections or synapses between ciliated cells and nerve fibers have not yet been identified, the assignment of sensory function to cells in the prosobranch osphradium is currently based solely on cytological observations and selective vital staining. Although the results of our and other studies have identified putative chemoreceptors, electrophysiological investigations will be necessary to confirm and elucidate structure-function relationships of receptor cells and specialized lamellar regions of the prosobranch osphradium.

Acknowledgments

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LITERATURE CITED

Alexander, C. G. 1970. The osphradium of *Conus flavidus*. *Mar. Biol.* **6**: 236–240.

Alexander, C. G., and M. W. Weldon. 1975. The fine structure of the osphradial leaflets in *Conus flavidus*. *Mar. Biol.* **33**: 247–254.

Altner, H., and L. Prillinger. 1980. Ultrastructure of invertebrate chemoreceptors, thermoreceptors and hygroreceptors and its functional significance. Pp. 69–140 In *International Review of Cytology*, Vol. 67, G. H. Bourne, and J. F. Danielli, eds. Academic Press, New York.

Bailey, D. F., and M. S. Laverack. 1966. Aspects of the neurophysiology of *Buccinium undatum*. I. Central responses to stimulation of the osphradium. *J. Exp. Biol.* **44**: 131–148.

Bailey, D. F., and P. R. Benjamin. 1968. Anatomical and electrophysiological studies on the gastropod osphradium. *Symp. Zool. Soc. Lond.* **23**: 263–268.

Brown, A. C., and R. G. Noble. 1960. Function of the osphradium in *Bullia*. *Nature* **188**: 1045.

Bullock, T. H., and G. A. Horridge. 1965. *Structure and Function in the Nervous System of Invertebrates*. W. H. Freeman and Co., San Francisco and London. 1610 pp.

Caprio, J., and R. Raderman-Little. 1978. Scanning electron microscopy of the channel catfish olfactory lamellae. *Tissue Cell* **10**: 1–9.

Crisp, M. 1973. Fine structure of some prosobranch osphradia. *Mar. Biol.* **22**: 231–240.

Davis, W. J., and E. M. Matera. 1982. Chemoreception in gastropod molluscs: Electron microscopy of putative receptor cells. *J. Neurobiol.* **13**: 79–84.

GARTON, D., AND W. B. STICKLE. 1980. Effects of salinity and temperature on the predation rate of *Thais haemastoma* on *Crassostrea virginica* spat. *Biol. Bull.* **158:** 49–57.

HACKNEY, C. M., C. R. MCCROHAN, AND S. J. HAWKINS. 1983. Putative sense organs on the pallial tentacles of the limpet, *Patella vulgata* (L.). *Cell Tissue Res.* **231:** 663–674.

HOLL, A. 1981. Marking of olfactory axons of fishes by intravital staining with Procion Brilliant Yellow. *Stain Technol.* **56:** 67–70.

MATERA, E. M., AND W. J. DAVIS. 1982. Paddle cilia (discocilia) in chemosensitive structures of the gastropod mollusk *Pleurobranchaea californica*. *Cell Tissue Res.* **222:** 25–40.

MUNN, E. A. 1974. *The Structure of Mitochondria.* Academic Press, New York. 465 pp.

NEWELL, P. F., AND A. C. BROWN. 1977. The fine structure of the osphradium of *Bullia digitalis* Meuschen (Gastropoda: Prosobranchia). *Malacologia* **16:** 197–205.

WELSCH, U., AND V. STORCH. 1969. Uber das Osphradium der prosobranchen Schnecken *Buccinium undatum* und *Neptunea antiqua*. *Z. Zellforsch.* **95:** 317–330.

YAMAMOTO, M. 1982. Comparative morphology of the peripheral olfactory organ in teleosts. Pp. 39–59 In *Chemoreception in Fishes: Development in Aquaculature and Fisheries Science*, Vol. 8, T. J. Hara, ed. Elsevier, New York.

Reference: *Biol. Bull.* **167**: 322–330. (October, 1984)

CHEMOSENSORY RESPONSES TO AMINO ACIDS AND CERTAIN AMINES BY THE CILIATE *TETRAHYMENA*: A FLAT CAPILLARY ASSAY

M. LEVANDOWSKY, T. CHENG, A. KEHR, J. KIM, L. GARDNER, L. SILVERN, L. TSANG, G. LAI, C. CHUNG, AND E. PRAKASH

Haskins Laboratories of Pace University, 41 Park Row, New York, New York 10038

Abstract

An assay for chemosensory responses by the ciliate *Tetrahymena thermophila* is described that uses glass capillaries with a rectangular cross-section (inner dimensions, $20 \times 2 \times 0.2$ mm). These have optical and geometrical properties permitting convenient observation of cell behavior within the capillaries.

Washed cells, starved for 12 h, accumulated preferentially in capillaries containing L-methionine, L-leucine, L-cysteine, L-histidine, L-histamine, cimetidine, agmatine, and berenil at concentrations of 10^{-3} *M* or less. They avoided capillaries containing tripelennamine, diphenhydramine, and pentamidine at these concentrations. It is argued that the actual response thresholds are much lower than the concentrations put into the capillaries, since cells respond to the gradient of the diffusing chemical.

L-Isoleucine, itself inert, blocked the response to L-leucine but not to L-methionine, L-cysteine, or L-histidine. L-Ethionine and l-homocysteine caused accumulation but not L-cysteine or DL-cystathionine. L-Cystine did not block the response to L-cysteine.

Cells accelerated when entering a capillary where accumulation occurred. On reaching the interior they swam more slowly and uniformly, and with fewer turns or stops than in control capillaries lacking the chemical signal, or when outside of the capillaries. Cells were inhibited from leaving both control and test capillaries, possibly because of accumulated wastes or secretions in the surrounding medium.

Introduction

Chemical senses have been analyzed extensively in bacteria (Adler, 1975; Koshland, 1980) and the cellular slime molds (Mato and Konijn, 1979), and are probably ubiquitous among motile microorganisms (Levandowsky and Hauser, 1978). Among ciliate protozoa, chemosensory responses have been much studied in *Paramecium* (Van Houten *et al.*, 1981) and *Blepharisma* (Miyake, 1980). The genus *Tetrahymena*, easily grown in chemically defined media and the subject of numerous genetic and biochemical studies, appears particularly promising for the analysis of the mechanisms of these responses. Csaba and Lantos (1973, 1975) demonstrated enhanced phagocytosis by starved *Tetrahymena* cells in the presence of low levels of serotonin and other vertebrate neurohormones. Ueda, Kobatake, and their colleagues (Ueda and Kobatake, 1977; Ataka *et al.*, 1978; Tanabe *et al.*, 1980) described dispersion or accumulation in response to a number of chemicals, particularly those producing a bitter taste in humans, and related these responses to interactions with membrane lipids and membrane potential as measured with fluorescent dyes. Almagor *et al.* (1981) adapted capillary methods which have been used with bacteria (Adler, 1975) and flagellates (Sjoblad *et al.*, 1978; Spero, 1984). They demonstrated responses to methionine and leucine, and related these to the influence of those chemicals on swimming behavior.

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We describe another assay method based on capillaries with a rectangular cross section. These have optical and geometrical properties which permitted detailed observations of behavior within them. We also extend the results of Almager *et al.* to include other amino acids and certain amines.

Materials and Methods

Organisms and culture methods

Tetrahymena thermophila (CU strain 307, mating type BIV) was obtained from Dr. Lea Bleyman and cultured at room temperature in autoclaved HTM medium: (gm/100 ml H_2O) dextrin 0.8; yeast autolysate (Difco) 0.5; liver concentrate (Sigma) 0.06; casein hydrolysate (Hycase, Sheffield) 0.6; morpholinopropane sulfonic acid (MOPS) 0.1; pH adjusted to 7.0 with NaOH.

Starvation pretreatment

Three to six day old cultures (late log phase, approximately 2×10^5 cells/ml) were harvested and washed twice by gentle centrifugation (2 min in a clinical centrifuge followed by resuspension in distilled H_2O), then recentrifuged and resuspended in starvation medium (S) (mg/100 ml H_2O): $CaCl_2$ $2H_2O$ 15.0; Na_2EDTA 60.0; N-tris (hydroxymethyl) methyl-glycine (TRICINE) 18.0; adjusted to pH 7.0 with NaOH. (A somewhat more complex medium, containing also magnesium, sodium, and potassium salts was used in early experiments, but later work showed these were not required for the chemosensory response). After starvation in S for at least 6 h, cells were washed once in distilled H_2O as before and resuspended in S and allowed to stand at least an hour before use in an experiment. During the starvation period the cells underwent metamorphosis to the faster-swimming dispersal form (Nelson and DeBault, 1978). In this assay significant chemosensory responses were seen only with the starved dispersal form, and never with well-fed cells.

The behavior of starved, washed cells appeared to be quite sensitive to the presence of trace contaminants. In preliminary studies it was found that the presence of Na_2EDTA or other organic ligands was desirable for normal swimming behavior. Salt solutions lacking this, made up with samples of glass-distilled water from six different laboratories of the New York–New Haven area, collected and transported in clean glass or plastic containers, resulted in abnormal swimming behavior such as swimming backward or in circles; this problem could be cured by addition of appropriate amounts of Na_2EDTA and $CaCl_2$ H_2O (see Discussion section).

Capillaries, glassware, and plasticware

Borosilicate glass capillaries ("microslides," Vitro Dynamics Inc., P. O. Box 285, Rockaway, NJ 07866) with rectangular cross-section and inner dimensions (mm) 20. $\times$ 2. $\times$ 0.2 were used. Before use, both new and used capillaries were cleaned by soaking overnight in concentrated H_2SO_4. Before and after soaking they were boiled in several changes of distilled H_2O. Capillaries cleaned in this manner yielded best results.

Glassware and plasticware used for these experiments were kept segregated and washed separately from other labware, using Alconox detergent and many rinses in hot water, followed by soaking in distilled H_2O. New or only slightly used plastic dishes tended to inhibit cell motility (see also Wolfe and Colby, 1981); this problem could be eliminated by soaking the dishes overnight in concentrated H_2SO_4, but concentrated HCl, NaOH 5 *N* or 10% Na_2EDTA were not effective (see Discussion).

Assay method

Capillaries were filled with S medium (control capillaries) or with S and a solute to be tested (test capillaries), by touching one end to the fluid, allowing the latter to be rapidly drawn in by capillarity. Properly cleaned capillaries fill very quickly. Capillaries were handled with clean forceps. Filled capillaries were then placed in a plastic dish with 40 mm inner diameter (the top half of a Corning 35-mm tissue culture dish) containing 3 ml of a cell suspension, diluted to approximately 25,000 cells/ml. Two capillaries, test and control, were placed in each dish and gently submerged to lie flat and parallel on the dish bottom, with ends separated by several mm from each other and from the sides of the dish. It should be noted that both ends of the capillary are open in this assay, in contrast to the capillary assays commonly used with bacteria (Adler, 1975).

Experiments were done at 28°C. Below 25°C the chemosensory response was never detected. Results of replicate experiments done in the dark and in various light regimes appeared to be the same.

After 5 min, capillaries were removed with forceps, gently blotted on the outside with tissue, and placed in a dry plastic dish. Behavior of organisms swimming inside the capillaries was observed with the microscope. Then the dish with the two capillaries was floated for 1 min on a hot water bath (65–75°C). The heat-killed cells in each capillary were then counted.

Six replicate assays of each concentration of the test chemical were done in each experiment. Experiments were then repeated several times with different cell preparations. Cells were also checked in a control experiment for response to 10^{-3} *M* L-methionine or L-histamine before being used to test other compounds. Purest available reagent grade commercial chemicals were used.

RESULTS

Statistical aspects.

To establish a statistical base line we did experiments in which no chemical cue was tested, but rather both capillaries in each dish contained only the salts solution S. Table I shows data for ten replicate experiments with the same cell preparation. These data appear inconsistent with a simple statistical model in which each cell "decides" independently of others whether to enter one or the other of the two identical capillaries. Such a model would predict a binomial distribution in which the great differences between the two capillaries in trials 1, 2, 3, 6, and 9 would be very improbable. This great variability may perhaps be due to cell interactions, in which case the assumption of statistical independence of the cells is not valid. Thus, standard parametric tests for significance are probably inappropriate.

Table II shows the effect of introducing 10^{-3} *M* L-methionine into one of the capillaries in each dish. Though there is great variability in both absolute numbers

TABLE I

Numbers of cells entering pairs of identical capillaries in replicate trials

Trial number	1	2	3	4	5	6	7	8	9	10
Capillary 1	228	34	164	78	75	238	120	158	135	212
Capillary 2	92	199	91	82	96	157	76	172	240	200

TABLE II

Response to L-methionine in six replicate trials

Trial number	1	2	3	4	5	6
Test capillary: S medium + 10^{-3} *M* Methionine	225	568	637	852	468	347
Control capillary: S medium	211	385	227	132	273	179

and ratios of cells in test and control capillaries, the former always had more cells than the latter in a given dish, and it is clear that they accumulated preferentially in the presence of 10^{-3} *M* methionine. By the nonparametric sign test, the probability of the data in Table II occurring by chance is less than .016. Because of high variability, of unknown origin, we adopted the sign test as a conservative measure of statistical significance, and each experiment was therefore done in six or more replicates. This was repeated with several different cell preparations before we accepted a response as significant.

Amino acid responses

Of 23 common amino acids tested, only four elicited significant responses by the above criterion (Table III). The L forms of methionine, leucine, histidine, and cysteine were active at 10^{-3}, 10^{-4}, and sometimes lower molar levels. Others tested and found inert were the L forms of alanine, arginine, asparagine, aspartic acid, cystine, glutamic acid, glutamine, glycine, isoleucine, lysine, ornithine, phenylalanine, proline, serine, taurine, threonine, tryptophan, tyrosine, and valine. In an abstract of preliminary results (Levandowsky *et al.*, 1982) we had reported responses to several of the latter also, but in subsequent experiments these were not significant by the above criterion.

TABLE III

Response to amino acids, amines, and their antagonists

Molar concentration	10^{-3}	10^{-4}	10^{-5}
Amino acids			
L-methionine	+	+	+*
L-histidine	+	+	+*
L-leucine	+	+	+*
L-cysteine	+	0	0
Amines			
L-histamine 2HCl	+	+	+*
Agmatine SO_4	+	+	+*
Histamine and diamine antagonists			
Cimetidine HCl	+	+	0
Berenil	–	+	+
Tripelenamine HCl	–	0	0
Diphenhydramine HCl	–	–	0
Pentamidine isethionate	–	–	0

+ = accumulation (more cells in test capillary).
– = dispersion (fewer cells in test capillary).
* = effect true only in some experiments.
0 = response not significant (see text).

Specificity of amino acid responses

Isoleucine was usually inert (in a few preparations slight accumulation occurred at 10^{-3} *M*). When added to the background, so that 10^{-3} *M* L-isoleucine was present in both control and test capillaries, and in the surrounding fluids, it blocked the response to leucine but not to methionine, cysteine, or histidine.

Several methionine analogs were tested. DL-Ethionine elicited a strong response in the same range as methionine, but not seleno-DL-methionine. DL-Homocysteine, a cysteine analog, was active in the same range as L-cysteine, but L-cystine and DL-cystathionine were inert. L-Cystine added to the background at 10^{-3} *M* did not block the response to either cysteine or methionine.

Histamine and the diamines

L-Histamine HCl (Table III) elicited a somewhat stronger aggregation response than histidine, over the same concentration range. Because of certain biochemical similarities, discussed below, we also tested a group of diamines. Of these, only agmatine proved active, causing aggregation in the same range as histamine and the four amino acids. Diaminopropane, putrescine, spermidine, spermine, and cadaverine were inactive.

Antihistamines and diamine antagonists

We examined several antihistamines, as well as anti-parasite drugs which are antagonists to both histamine and polyamines (see Discussion). Of these, the H-2 antihistamine cimetidine and the trypanocide Berenil (diminazene aceturate) elicited aggregation at 10^{-3} *M*. Dispersion (fewer cells in the test capillary) occurred with the H-1 antihistamines tripelennamine and diphenhydramine, and with the polyamine antagonist pentamidine.

Behavioral basis of the responses

Using an ocular micrometer and a stopwatch we measured swimming speed and turning frequency of individual cells selected arbitrarily by following the first cell to swim past a line on the ocular micrometer grid in a given time. Tables IV and V show differences in swimming behavior in the test capillary and elsewhere.

A basic element in the repertoire of swimming behavior of starved *T. thermophila* cells, and the most important one for getting cells into the capillary during a five minute experimental period, consists simply of straight or nearly straight "runs" punctuated by turns. Unlike *Paramecium, T. thermophila* does not usually stop, but often slows down during the turn.

On entering a test capillary containing an attractant, cells immediately accelerated and swam rapidly without turns for 4–8 mm. On reaching the midregion of the

TABLE IV

Average swimming speed (mm/s, $\pm 2\sigma$) in a typical experiment

1. Midregion of test capillary	.44 ± 0.3 (n = 10)
2. Entering the test capillary	.72 ± 0.4 (n = 10)
3. Midregion of control capillary	.58 ± 0.4 (n = 10)
4. Entering control capillary	.50 ± .03 (n = 10)
5. Outside capillaries	.49 ± .05 (n = 10)

TABLE V

Average time between turns or stops (s, ± 2σ) in a typical experiment

Middle of test capillary	8.2 ± 1.2 (n = 20)
Middle of control capillary	4.8 ± 1.1 (n = 20)
Outside of capillaries	4.2 ± 1.0 (n = 20)

capillary they swam more slowly and uniformly than elsewhere, with fewer turns than in the control capillary or outside of the capillaries (Tables IV, V). On attempting to leave, cells in both the test and control capillaries tended to stop and turn more frequently in a zone near the end of the capillary, and in some cell preparations appeared virtually unable to leave once they had entered either the test or the control capillary.

When cells inside the capillary swam into the wall they usually just "reflected" like billiard balls, at an equal angle, without slowing down. These were not counted as turns in the observations.

In summary: outside the test capillary, where there was no added chemical cue, the cells moved in a series of small, relatively fast "runs." There was a large variability in individual speed and turning frequency, and the overall impression was of a jerky, erratic searching behavior. At the entrance to the test capillary, where there is a gradient of diffusing chemical signals, they suddenly and dramatically accelerated and swam up the gradient to the midregion of the capillary. This appears to be the main factor causing greater accumulation in the test capillary than in the control during the five minute duration of the experiment. In the midregion, where the chemical concentration was presumably constant, they slowed down and turned less frequently than outside, and the overall impression was of a very uniform, even motion, with few turns or stops. On attempting to swim out of either test or control capillary, their motion became jerky and irregular, with many stops and turns. In some cell preparations they seemed to be unable to leave. Since this occurred also in the control capillary, this was probably the effect of wastes or cell secretions in the fluid outside, which had cells in it for at least an hour before the experiment began.

DISCUSSION

Responses to methionine and leucine were also studied by Almagor *et al.* (1981), using the WH-52 strain of *T. thermophila* and a somewhat different capillary assay method, as well as microscopic observation to record motility in various attractant concentrations. The latter method detected responses at much lower levels than the capillary method. As they note, organisms responding to a gradient of a diffusing chemical would initially encounter a much lower concentration than that originally placed in the capillary. Thus capillary assays, though convenient and meaningful, are relatively insensitive and overestimate the response threshold by at least an order of magnitude. Similar conclusions have been drawn regarding capillary assays of bacterial chemosensory assays (Hazelbauer and Adler, 1971). Our capillary assays and those of Almagor *et al.* appear to be similar in sensitivity.

Ordinary capillaries with a circular cross section present difficulties however, when they are submerged in the cell suspension it is difficult to determine whether cells swimming at a given focal depth are inside the capillaries. Furthermore, when they are removed from the medium, fluid-filled capillaries act as lenses, and cells swimming

inside them are extremely difficult to watch with the microscope. These problems do not arise with the rectangular capillaries used in this assay.

The abnormal behavior in the absence of organic ligands such as Na_2EDTA, noted in the materials and methods section, may be due to heavy metals leaching from glass or plastic containers (Bernhard, 1977). Inhibition of cell motility (sticking to the bottom) in new, untreated plastic dishes probably stems from surface charge-related hydrophobic interactions between cells and the plastic surface (Kitamura, 1982; D. Rittshoff, pers. comm.).

It would be interesting to know whether the WH-52 strain used by Almagor *et al.* also responds to cysteine, histidine, and the amines listed in Table III. There is a great deal of biochemical variation among morphologically indistinguishable members of this group (Nanney, 1980), and one would like to know whether sensory responses are a conservative or a variable feature. As noted above, we had indications in early experiments of responses to other amino acids, notably tyrosine, phenylalanine, arginine, and serine, but these did not appear consistently in subsequent experiments. Such inconsistency could have various explanations, one of which is a latent sensitivity to these compounds that is not always expressed. This deserves further study.

Amines and amino acids may serve as useful ecological signals for food. Thus, Fuzessery *et al.* (1978) suggested a correlation between the spiny lobster's chemosensitivity to very low levels of taurine and the latter's particular value as a potential food indicator in the marine environment. In the case of *Tetrahymena,* a freshwater phagotroph, there are numerous laboratory studies of feeding behavior (Nilsson, 1979), but virtually no field studies of its natural history—its natural diet is, strictly speaking, unknown. In the lab it is usually grown on living or dead bacteria or yeast, or in defined or undefined liquid media containing precipitated particles. Histamine is a common waste product of bacterial decomposition, particularly of plant tissues (Guggenheim, 1951), and agmatine is an amine restricted to certain plants and bacteria. Beyond these hints, we have no clues yet regarding the adaptive value of responses to this particular set of amino acids and amines.

From a comparative, phylogenetic point of view the histamine response is of particular interest. Csaba and Lantos (1975) found that low levels of histamine and two histamine antagonists also stimulated phagotrophy in their GL strain. The positive (accumulation) response to the drug cimetidine, used to block the H-2 histamine receptor in treating ulcers, probably reflects its structural similarity to histamine (the presence of an imidazole group).

Tripelennamine and diphenhydramine on the other hand, drugs used to block histamine H-1 receptors in treating allergy and cold symptoms, gave rise to a negative (dispersion) response. Though they are antihistamines, these do not resemble histamine structurally and lack the imidazole group, but do have pharmacological and chemical affinities to local anesthetics of the cocaine family. The mode of action of the latter is thought to involve, among other possibilities, alteration of the physical properties of the cell membrane. We think it likely that some chemosensory responses of *Tetrahymena* will prove to be due to binding of the signal molecule to specific membrane-bound or intracellular receptor molecules, probably proteins, but some responses may be simply due to non-specific changes in physicochemical properties of the membrane. This was indicated by studies in which negative (repulsion) responses to various hydrophobic chemicals and to bitter substances were correlated with changes in membrane fluidity (Ataka *et al.,* 1978; Tanabe *et al.,* 1980).

Berenil (diminazene aceturate) and pentamidine are known mainly as anti-trypanosomal drugs. It appears that these and a number of other anti-trypanosomal and anti-malarial drugs are inhibitors of both histamine N-methyl transferase and also

diamine oxidase (Duch *et al.*, 1984). This link between histamine and the diamines is not yet understood, but appears promising as a pharmacological principle. Further analysis of the *Tetrahymena* response to such compounds would be desirable and might yield useful information on clinically important questions.

Chemosensory responses to amino acids are phylogenetically widespread, being found in bacteria (Adler, 1975; Koshland, 1980), algae (Hauser *et al.*, 1975; Sjoblad *et al.*, 1978), many invertebrates (*e.g.*, Ache, 1972) and vertebrates (*e.g.*, Caprio, 1978). In catfish, L-cysteine was the most effective olfactory stimulus tested (Caprio, 1978). Amino acids also act as excitatory transmitters in the brain and may be involved in some forms of epilepsy (Croucher *et al.*, 1982). In particular, L-cysteine may function as a transmitter in the brain (Watkins and Evans, 1981).

We have also found responses by *T. thermophila* to several other chemical groups, including neurotransmitters and hormones. Preliminary accounts of this work and of studies of the ionic requirements of the chemosensory response have appeared (abstracts: Levandowsky *et al.*, 1982; Gardner and Levandowsky, 1983; Tsang and Levandowsky, 1983).

The possibility of homologies between *Tetrahymena* responses to amino acids and amines, and those in higher organisms is intriguing. In addition to its evolutionary interest, this would suggest the practical possibility of using this organism as a model system to investigate chemosensory mechanisms, profiting from the ease with which it can be grown in mass culture, and the possibilities of genetic analysis.

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LITERATURE CITED

ACHE, B. W. 1972. Amino acid receptors in the antennules of *Homarus americanus*. *Comp. Biochem. Physiol.* **42:** 807–811.

ADLER, J. 1975. Chemotaxis in bacteria. *Ann. Rev. Biochem.* **44:** 341–366.

ALMAGOR, M., A. RON, AND J. BAR-TANA. 1981. Chemotaxis in *Tetrahymena thermophila*. *Cell Motility* **1:** 261–268.

ATAKA, M., A. TSUCHII, T. UEDA, K. KURIHARA, AND Y. KOBATAKE. 1978. Comparative studies on the reception of bitter stimuli in the frog, *Tetrahymena*, slime mold and *Nitella*. *Comp. Biochem. Physiol.* **61A:** 109–115.

BERNHARD, M. 1977. Chemical contamination of culture media: assessment, avoidance and control. Pp. 1459–1499 in *Marine Ecology*, Vol. III, part 3, O. Kinne, ed. Wiley & Sons, New York.

CAPRIO, J. 1978. Olfaction and taste in the channel catfish: an electrophysiological study of the responses to amino acids derivatives. *J. Comp. Physiol.* **123:** 357–371.

CROUCHER, M. J., J. F. COLLINS, AND B. S. MELDROM. 1982. Anticonvulsant action of excitatory amino acids. *Science* **216:** 899–901.

CSABA, G., AND T. LANTOS. 1973. Effect of hormones on protozoa. Studies on the phagocytotic effect of histamine, 5-hydroxytryptamine and indolacetic acid in *Tetrahymena*. *Cytobiologie* **7:** 311–315.

CSABA, G., AND T. LANTOS. 1975. Specificity of hormone receptors in *Tetrahymena*. Experiments with serotonin and histamine antagonists. *Cytobiologie* **11:** 44–49.

DUCH, D. S., C. J. BACCHI, M. P. EDELSTEIN, AND C. A. NICHOL. 1984. Inhibitors of histamine metabolism *In vivo* and *In vitro:* correlations with anti-trypanosomal activity. *Biochem. Pharmacol.* **33:** 1549–1553.

FUZESSERY, Z. M., W. E. S. CARR, AND B. W. ACHE. 1978. Antennulary chemosensitivity of the spiny lobster, *Panulirus argus:* studies of taurine sensitive receptors. *Biol. Bull.* **154:** 226–240.

GARDNER, L., AND M. LEVANDOWSKY. 1983. Ca^{++} dependence of chemosensory response and swimming speed in *Tetrahymena thermophila*. *J. Protozool.* **30:** 13A (Abstr.).

GUGGENHEIM, M. 1951. *Die biogenen Amine.* S. Karger, Basel. 619 pp.

HAUSER, D. C. R., M. LEVANDOWSKY, S. H. HUTNER, L. CHUNOSOFF, AND J. S. HOLLWITZ. 1975. Chemosensory responses by the heterotrophic marine dinoflagellate *Crypthecodinium cohnii. Microb. Ecol.* **1:** 246–254.

HAZELBAUER, G. L., AND J. ADLER. 1971. Role of the galactose binding protein in chemotaxis of *Escherichia coli* toward galactose. *Nature* **230:** 101–104.

KITAMURA, A. 1982. Attachment of *Paramecium* to polystyrene surfaces: a model system for the analysis of sexual cell recognition and nuclear activation. *J. Cell Sci.* **58:** 185–199.

KOSHLAND, JR., D. E. 1980. *Bacterial Chemotaxis as a Model Behavioral System.* Raven Press, New York. 193 pp.

LEVANDOWSKY, M., T. CHANG, A. KEHR, J. KIM, L. GARDNER, L. SILVERN, AND I. R. LAPIDUS. 1982. Chemosensory responses of *Tetrahymena:* a flat capillary assay. *J. Protozool.* **29:** 498 (Abstr.).

LEVANDOWSKY, M., AND D. C. R. HAUSER. 1978. Chemosensory responses of swimming algae and protozoa. *Int. Rev. Cytol.* **53:** 145–210.

MATO, J. M., AND T. M. KONIJN. 1979. Chemosensory transduction in *Dictyostelium discoideum.* Pp. 181–221 in *Biochemistry and Physiology of Protozoa,* 2nd Ed., Vol. 2, M. Levandowsky and S. H. Hutner, eds. Academic Press, NY.

MIYAKE, A. 1981. Physiology and biochemistry of conjugation in ciliates. Pp. 125–198 in *Biochemistry and Physiology of Protozoa,* 2nd Ed., Vol. 4, M. Levandowsky and S. H. Hutner, eds. Academic Press, NY.

NANNEY, D. L. 1980. *Experimental Ciliatology.* Wiley and Sons, New York. 304 pp.

NELSEN, E. M., AND L. DEBAULT. 1978. Transformation in *Tetrahymena pyriformis:* description of an inducible phenotype. *J. Protozool.* **23:** 113–119.

NILSSON, J. R. 1979. Phagotrophy in *Tetrahymena.* Pp. 339–380 in *Biochemistry and Physiology of Protozoa,* 2nd Ed., Vol. 2, M. Levandowsky and S. H. Hutner, eds. Academic Press, NY.

SJOBLAD, E. D., I. CHET, AND R. MITCHELL. 1978. Chemoreception in the green alga *Dunaliella tertiolecta. Curr. Microbiol.* **1:** 303–307.

SPERO, H. J. 1984. Chemosensory behavior in the phagotrophic dinoflagellate *Gymnodium fungiforme. J. Phycol.* (In press.)

TANABE, R., K. KURIHARA, AND Y. KOBATAKE. 1980. Changes in membrane potential and membrane fluidity in *Tetrahymena pyriformis* in association with chemoreception of hydrophobic stimuli: fluorescence studies. *Biochemistry* **19:** 5339–5343.

TSANG, L., AND M. LEVANDOWSKY. 1983. Cholinergic chemosensory responses of *Tetrahymena. J. Protozool.* **30:** 13A (Abstr.).

UEDA, T., AND Y. KOBATAKE. 1977. Hydrophobicity of biosurfaces as shown by chemoreceptive thresholds in *Tetrahymena, Physarum* and *Nitella. J. Membrane Biol.* **34:** 351–368.

VAN HOUTEN, J., D. C. R. HAUSER, AND M. LEVANDOWSKY. 1981. Chemosensory behavior in protozoa. Pp. 67–124 in *Biochemistry and Physiology of Protozoa,* 2nd Ed., Vol. 4, M. Levandowsky and S. H. Hutner, eds. Academic Press, NY.

WATKINS, J. C., AND R. H. EVANS. 1981. Excitatory amino acid transmitters. *Ann. Rev. Pharmacol. Toxicol.* **21:** 165–204.

WOLFE, J., AND R. H. COLBY. 1981. A method for immobilization of living *Tetrahymena. Exp. Cell Res.* **134:** 313–317.

Reference: *Biol. Bull.* **167**: 331–338. (October, 1984)

TRANSFER OF NEMERTEAN EGG PREDATORS DURING HOST MOLTING AND COPULATION

DANIEL E. WICKHAM[1], PAMELA ROE[2], AND ARMAND M. KURIS[3]

[1]*Bodega Marine Laboratory, Bodega Bay, California 94923,* [2]*Department of Biology, California State College Stanislaus, Turlock, California 95380, and* [3]*Marine Science Institute and the Department of Biological Sciences, University of California, Santa Barbara, California 93106*

ABSTRACT

Juvenile nemertean egg predators were able to efficiently transfer from the premolt cuticle to the postmolt cuticle of male and female crabs when the host molted. These worms also efficiently transferred from male to female hosts at copulation. The synchronized responses of the nemertean worms to host physiology and behavior dramatically concentrate the nemertean population on the sole food source required for worm reproduction: crab eggs. The efficient location of reproductive crabs by juvenile worms increased the likelihood that these worms can have significant effects on crab fisheries when worm population density is high.

INTRODUCTION

Worms of the nemertean genus *Carcinonemertes* are ectosymbionts that feed on the eggs of decapod crustaceans. Infestation of hosts follows a planktonic larval period. The juvenile worms then ensheath in various protected spots on the host exoskeleton (Humes, 1942; Kuris, 1978; Wickham, 1980). These quiescent worms apparently subsist on dissolved organic matter leaked from crab arthrodial membranes and absorbed through the tegument of the worms (Roe *et al.*, 1981; Crowe *et al.*, 1982). *Carcinonemertes errans* infests the dungeness crab, *Cancer magister,* and will settle on crabs of any age or sex (Wickham, 1980). This crab is long lived and females produce their first egg clutch when two or three years old (Butler, 1961). Mating occurs only at the female molt. Males sequester premolt females and copulation occurs immediately following ecdysis of the female (Snow and Nielson, 1966). They reproduce once a year, brood eggs for about 90 days in central California, and must molt before producing subsequent broods. Worms infesting this crab species must feed on crab eggs in order to reproduce themselves.

In 1974 the average burden of nemerteans on ovigerous female dungeness crabs in central California was 29,000 *C. errans* per crab causing the mortality of over 50% of the crab eggs produced in the San Francisco region (Wickham, 1979). This level of infestation still persists and has shown no evidence of decline even though host abundance has been dramatically reduced relative to historical levels for this entire time period. Nemertean abundance on dungeness crabs from northern California, Oregon, and Washington has increased from 1000–2000 in 1974 and 1975 to levels comparable to those found in central California with a concomitant increase in crab egg mortality, possibly in response to record high populations of host crabs followed by a drastic cyclic decline (Wickham, 1980; unpubl.)

Nemerteans have been observed on many species of decapod crustaceans, occasionally with large numbers of worms on individual hosts (Humes, 1942; Aiken *et*

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al., 1983) but never to the extent found on *Cancer magister*. The only other instance of widespread infestations in epidemic proportions by nemerteans is on the Alaskan king crab, *Paralithodes camtschatica*. Populations over much of the range of this crab are currently suffering dramatic brood wastage apparently related to a marked increase in the abundance of nemerteans which are either new to this host, or, more likely were not observed previously on this heavily studied host because of typically low numbers (Wickham and Kuris, unpubl.) Factors which allow the maintenance of such large densities of ectosymbionts on hosts which periodically molt and have been thought to shed their symbionts at the molt (Humes, 1942) must be identified to understand the role of these organisms in their hosts' dynamics.

Materials and Methods

Specimens of juvenile and mature *Cancer magister* with resident populations of *Carcinonemertes errans* were collected from the waters near Bodega Bay, California. Mature male and female crabs which showed evidence of approaching molt were held separately in laboratory tanks and monitored through the molt. Worm distributions over the host exoskeleton were assayed by enumerating worms at a representative selection of sites normally occupied by worms. These included walking legs, axillae, chelipeds, thorax, abdomen, pleopods (female), or copulatory appendages (male). Photographs were taken to display worm disposition at the time of ecdysis. Worms were counted on the exuviae after molt. Redistribution of the worms on the new exoskeleton was followed over the first few days following molting by sampling the original sites. An exhaustive census of worms on the new exoskeleton was then conducted to determine the proportion of the original population which transferred at molt.

Intermolt transfer was also observed on juvenile crabs held in cubicles in plastic parts boxes. These crabs had low numbers of worms and, in 7 of 12 instances, worm populations on them were augmented by the addition of worms to the exoskeleton prior to ecdysis. The additional worms were obtained from adult male crabs. Worms crawled onto juvenile crabs placed with the worms in finger bowls. Observations of worm behavior at host ecdysis were conducted only after an acclimatization period of approximately a week.

Transfer of worms to females at mating was observed during two mating instances in two fashions. In the first instance the exoskeleton of a female was cleaned by sparging with 95% ethanol until all worms were killed and removed. She was then mated with an infested male and the number of worms on her exoskeleton was counted the following day and compared with the number remaining on the male to determine the proportion transferred. In the second instance an infested female was mated with a male whose worms had been vitally stained with neutral red dye. The distribution of these stained worms was then noted on the female the day after mating and the number was again compared with the number remaining on the male.

Observations were also made with *C. epialti* worms on the crab *Hemigrapsus oregonensis*. The proportion of worms transferring at molt was measured in the same fashion as with *C. magister*.

Results

When mature female *Cancer magister* molt, an average of 88% of the resident worms migrate to the new exoskeleton (Table I). The manner of migration is active, directed and strikingly illustrated in Figure 1. Several days prior to host ecdysis worms become more concentrated near the base of the abdomen where they have greater

TABLE I

Numbers of Carcinonemertes errans *on adult female crabs and exuviae after molting*

Crab No.	No. worms on exuvium	No. worms on new crab	Total no. worms	Proportion of worms transferring
1	134	1891	2025	.934
2	277	899	1166	.762
3	89	1612	1701	.948
4	827	5536	6363	.870
				$\bar{x}$ = .88
				S.D. = .09

access to the epimeral suture. Worms crawl toward the epimeral suture when decalcification begins. By the time the suture opens most of the worms are massed against the new exoskeleton of the crab so they can remain with the crab as the exuvium is shed.

Shortly after ecdysis worms can be observed migrating forward and outward until they assume a typical distribution pattern (see Table IV). During intermolt periods worms tend to be contracted and clustered in groups. On the early post-ecdysial crab, worms are elongated and can often be seen moving across calcified portions of the exoskeleton where they normally are not found. At this time worms can often be found head to tail in a single file along suture lines in the axils.

Transfer at ecdysis on male crabs appears to be more variable than on females.

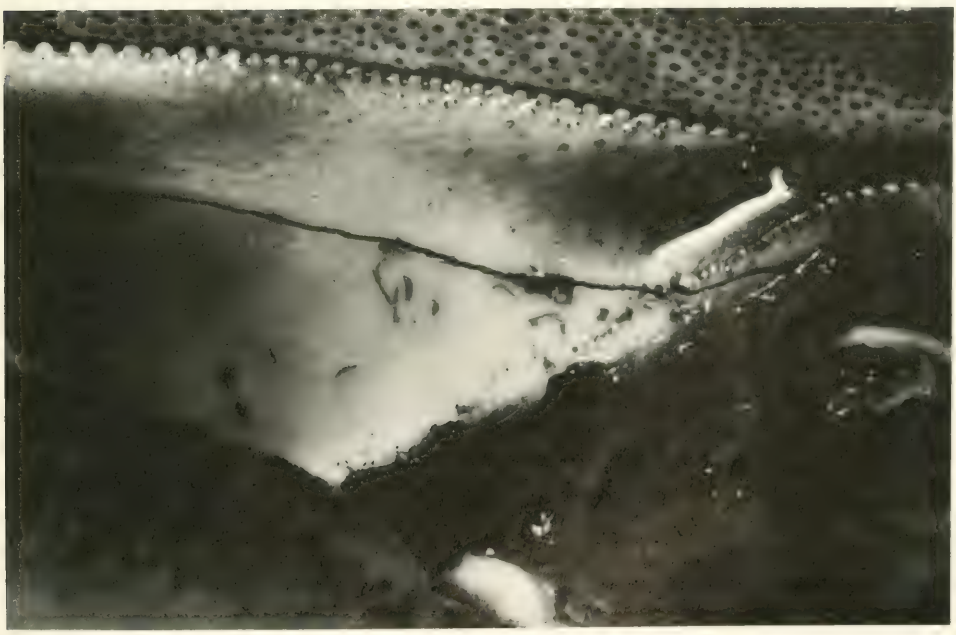

FIGURE 1. Worms actively crawling toward decalcifying epimeral suture on postero-lateral surface of carapace.

TABLE II

Numbers of Carcinonemertes errans *on adult male crabs and exuviae after molting*

	Crab no.	No. worms on new crab	No. worms on exuvium	Total no. worms	Proportion of worms transferring
Spring	1	22	3	25	.12
	2	14	5	19	.26
	3	24	3	11	.27
	4	145	27	145	.16
	5	124	22	146	.15
					$\bar{x}$ = .19
					S.D. = .06
Late summer	1	25	377	402	.94

During spring when male crabs carried lower worm burdens most worms were lost with the molt. Worms on the male crab which molted in late summer transferred with an efficiency comparable to those on female crabs (Table II).

On juvenile crabs worms transferred on both sexes (Table III). Worms disappeared from the system in these experiments. An average of only 22% of the premolt worms were found on both the post molt and exuviae with male crabs (n = 6) and 73% of the premolt worms found with female (n = 9) post molt and exuviae. Comparable figures for loss to the system on adult crabs are not available given the difficulty of quantifying total worm abundance on pre-molt mature female crabs without first sacrificing the crab.

During the two mating instances approximately 90% and 85% respectively of the worms from the male transferred to the female (400 of approximately 5000, and 600 of approximately 3200 worms were left on the males after mating). In the observations done on worms dyed with neutral red the worms from males were distributed in a different pattern than the resident worms (G-test, $P < .01$). They tended to be slightly more concentrated under the abdomen (thoracic sutures plus bases of pleopods) than the preexisting population of worms on the female (Table IV).

The typical distribution of juvenile worms on intermolt males was much more concentrated in sites under the abdominal flap than on intermolt females (Table V).

Worms on the host *Hemigrapsus oregonensis* transferred in a fashion similar to that found with worms on *Cancer magister* but with somewhat less efficiency. Worms transferred more efficiently on male crabs (Table VI).

DISCUSSION

The striking behavior exhibited by *Carcinonemertes errans* on the crab *Cancer magister* at the molt represents an important adaptation for increasing the likelihood

TABLE III

Average number of Carcinonemertes errans *on premolt and postmolt juvenile* Cancer magister *and their exuviae (±S.D.)*

	Crab sex	Average no. worms on exuviae	Average no. worms on new crab	Average proportion transferring
M	(n = 7)	.57 (±.8)	7.43 (±4.8)	.93 (±.1)
F	(n = 5)	.60 (±1.3)	4.40 (±2.1)	.94 (±.1)

TABLE IV

Distribution of worms dyed with neutral red compared to undyed worms on adult female crab after mating

Site on crab	No. dyed worms	No. undyed worms	Proportion of total dyed	Proportion of total undyed
Chela	2	16	.01	.03
Coxal-basis joint on walking leg	35	101	.13	.17
Merus-carpus joint on walking leg	9	37	.03	.06
Thoracic sutures under abdomen	155	235	.59	.40
Bases of pleopod	60	192	.23	.33

of eventual arrival on a crab egg clutch where worms can complete their life cycle. The manner of movement by worms prior to actual ecdysis suggests that worms receive some type of preliminary information which causes them to begin to move toward areas with more direct access to the epimeral suture. The normal location of worms on males suggests that there is some recognition system involved in site selection. Juvenile worms on females can be found in virtually any protected crevice on the ventral surface of the body and on the limbs. On males most juvenile worms are under the folded abdomen near the copulatory appendages. This obviously facilitates transfer to females at mating but it also suggests that worms can distinguish male from female hosts and modify their site of infestation accordingly.

The nature of the signal for nemertean concentration near the abdomen just prior to ecdysis is unknown. Juvenile *C. errans* occur on or near arthrodial membranes which are known to be "leaky" (Crowe *et al.*, 1982). Worms actively absorb dissolved organic matter being leaked by the host at these sites (Roe *et al.*, 1981) so it is possible that alteration of the chemical nature of worm infestation sites provides a cue. One event which occurs at the time of molting is the separation of the epicuticle from the newly forming cuticle below, which could possibly alter the rate of leakage through

TABLE V

Distribution of juvenile worms on intermolt host exoskeletons (±S.D.)

	Male (n = 6)		Female (n = 3)	
Site	No. worms	Proportion of total worm pop.	No. worms	Proportion of total worm pop.
Axillae	24.0 (±13.7)	.06	167.3 (±16.7)	.12
Walking legs	10.3 (±4.4)	.02	891.3 (±276.3)	.61
Chelipeds	0.0	.00	6.7 (±5.8)	.01
Thorax	141.3 (±21.2)	.33	54.3 (±28.2)	.04
Abdomen	183.8 (±33.1)	.43	133.7 (±73.5)	.09
Pleopods or copulatory appendages	65.0 (±32.5)	.15	200.0 (±130.0)	.13
Proportion under abdomen	(.92) G test, $P < .01$		(.26)	

TABLE VI

Proportion of worms transferring to the new molt on male and female Hemingrapsus oregonensis *(±S.D.)*

Sex	No. worms on exuvium	No. worms on new crab	Average proportion transferring
M (n = 7)	0.14 (±.38)	1.71 (±.76)	.95 (±.12)
F (n = 12)	2.25 (±1.5)	3.00 (±4.2)	.46 (±.35)

the membranes or worms could sense molting fluid present between old and new cuticle.

The juvenile nemerteans on a pre-molt adult crab appear to aggregate where they have access to the epimeral suture but it is not until the suture actually begins to open that they migrate out across open calcified carapace to reach the suture. Again the chemicals which might be the actual attractants are unknown. Clearly the decalcification occurring there is a unique chemical event on the host and any number of compounds such as enzymes or breakdown products could be utilized as a cue for worm migration. The process of transfer on molting female crabs appears to be a multi-stage process involving recognition of diverse agents and specific action patterns. In contrast the shift from male to females at mating may be a one-stage process cued by the ecdysis of the female crab. Males mate with females just after the female has shed her exoskeleton and it is then that transfer from the male to female by worms occurs. In the transfer of worms marked with neutral red, worms from the male were more concentrated under the female abdomen relative to the distribution of the worms already on the female. It is not known if the male-derived worms would eventually have spread out similar to the worms already there.

The different behavior of worms on male crabs that molt before and after the spring mating season is consistent with our other observations and reinforces the adaptive behavior for *C. errans.* The one example of efficient transfer occurred on a male in late summer, several months after the mating season which is usually in February or March in central California. Worms on males transferring at this time become available for venereal transmission the next breeding season. Male crabs in early spring had a mean burden of 129.4 worms (n = 23). In contrast in the fall male crabs carried 1603.5 worms each (n = 29). The lower burden just after the mating season is consistent with the observation of venereal transfer.

The inability of a high proportion of worms to transfer at ecdysis on male crabs that molt immediately after the mating season is intriguing. Perhaps most of the remaining worms represent remnant population that were either unresponsive to previous transfer stimuli or are located in sites relatively inaccessible to such stimuli.

In the experiment involving transferral of worms on juvenile *Cancer magister* a large proportion of the worms disappeared and were found on neither the new crab cuticle nor on the exuvium. Most of this loss to the system occurred on male juvenile hosts but these were the hosts which had worms placed on them because of low original worm burdens. Perhaps acclimatization to the new host was insufficient resulting in worm departure. Worm transfer at molt was highly efficient in the worms which stayed in the experimental chambers.

The ability to transfer and concentrate on mature female crabs is highly adaptive for *C. errans.* It is only after feeding on host eggs that the worm can complete its own life cycle (Wickham, 1980). This transfer ability coupled with the fact that *C. errans* appears to be able to survive on amino acids leaked by the host for an indefinite

period (Roe *et al.*, 1981) means that once a larval worm finds a host it has a relatively high probability of eventually feeding on crab eggs.

The fact that *Carcinonemertes epialti* on the host *Hemigrapsus oregonensis* also is able to transfer at host ecdysis suggests that this behavior is general for *Carcinonemertes.* One other worm has been observed to transfer at host ecdysis on the host *Pinnixia tubicula* (J. McDermott, pers. comm.). Humes (1942) noted that *C. carcinophila* was shed with the exuvium when the host *Callinectes sapidus* molted. The location of worms on non-ovigerous hosts in the Atlantic differs in one important respect, however. *Carcinonemertes carcinonphila* is generally found sheathed between the gill lamellae of its host when not on an ovigerous crab (Humes, 1942; Hopkins, 1947). *Pseudocarcinonemertes homari* also lives in the branchial chamber and on the gills of the lobster *Homarus americanus* (Fleming and Gibson, 1981). On the Pacific coast, nemerteans have never been found in any number on the host gills (Wickham, 1978; unpubl.). It is possible that the location of the juvenile worms prevented transfer at molt, but in the case of the host *Pinnixa tubicola* the worms which transferred also lived in the host branchial chamber (J. McDermott, pers. comm.). Our observations of transfer by *C. errans* occurred because of the unusually high densities of worms involved. This behavior only became obvious when vast numbers of worms were observable. Further studies on transfer at molt by *Carcinonemertes carcinophila* are warranted.

The most significant consequence of these findings is *C. errans* has a heretofore unsuspected ability to accumulate on ovigerous crabs. This worm has been an enigma in that in central California crabs can carry as many as 100,000 worms. Yet *C. errans* has a fecundity on the order of 1000 eggs per year (Wickham, 1980) which is quite low when compared to other planktonically dispersed marine organisms. Efficiency in finding and remaining with hosts is critical to their success.

Thus, the predatory impact of *C. errans* on crab reproduction is maximized. Since the commercial season (for male dungeness crabs only) follows the channelization of the worm population to the reproductive female crabs, only a very small fraction of the worm population is lost to the principal source of crab mortality: the fishery. While crab populations may be temporarily depressed due to fishing, the worm population is buffered by the relative longevity of their unfished female hosts. The long-term consequences of the complex but efficient life cycle of *C. errans* for the dungeness crab fishery must now be explored.

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LITERATURE CITED

AIKEN, D. E., S. L. WADDY, L. S. UHAZY, AND A. CAMPBELL. 1983. A nemertean destructive to the eggs of the Lobster, *Homarus americanus. Rapp. P.-v. Reun. Cons. Int. Explor. Mer.* **182:** 130–133.

BUTLER, T. H. 1961. Growth and age determination of the Pacific edible crab, *Cancer magister* Dana. *J. Fish. Res. Board Can.* **18:** 873–891.

CROWE, J. H., L. M. CROWE, P. ROE, AND D. E. WICKHAM. 1982. Uptake of DOM by nemertean worms: association of worms with arthrodial membranes. *Am. Zool.* **22:** 671–682.

FLEMING, L. C., AND R. GIBSON. 1981. A new genus and species of monostiliferons hoplonemertean, ectohabitant on lobsters. *J. Exp. Mar. Biol. Ecol.* **52:** 79–93.

HOPKINS, S. 1947. The nemertean *Carcinonemertes* as an indicator of the spawning history of the host, *Callinectes sapidus. J. Parasitol.* **33:** 146–150.

HUMES, A. G. 1942. The morphology, taxonomy and binomics of the nemertean genus *Carcinonemertes. Ill. Biol. Monogr.* **18:** 1–105.

KURIS, A. M. 1978. Life cycle, distribution, and abundance of *Carcinonemertes epialti,* or nemertean egg predator of the shore crab, *Hemigrapsus oregonensis* in relation to heart size, reproduction, and molt cycle. *Biol. Bull.* **159:** 247–257.

ROE, P. 1979. Aspects of development and occurrence of *Carcinonemertes epialti* (Nemertea) from shore crabs in Monterey Bay, California. *Biol. Bull.* **156:** 130–140.

ROE, P., J. H. CROWE, L. M. CROWE, AND D. E. WICKHAM. 1981. Uptake of amino acids by juveniles of *Carcinonemertes errans* (Nemertea). *Comp. Biochem. Physiol.* **69:** 423–427.

SNOW, C. D., AND J. R. NIELSON. 1966. Premating and mating behavior of the Dungeness crab (*Cancer magister* Dana). *J. Fish. Res. Board Can.* **23:** 1319–1323.

WICKHAM, D. E. 1978. A new species of *Carcinonemertes* (Nemertea: Carcinonemertidae) with notes on the genus from the Pacific Coast. *Proc. Biol. Soc. Wash.* **91:** 197–202.

WICKHAM, D. E. 1979. Predation by the nemertean *Carcinonemertes errans* on eggs of the Dungeness crab, *Cancer magister. Mar. Biol.* **55:** 45–53.

WICKHAM, D. E. 1980. Aspects of the life history of *Carcinonemertes errans* (Nemertea: Carcinonemertidae) an egg predator of the crab *Cancer magister. Biol. Bull.* **159:** 247–257.

Reference: *Biol. Bull.* **167**: 339–353. (October, 1984)

CHEMICAL MEDIATION OF APPETITIVE FEEDING IN A MARINE DECAPOD CRUSTACEAN: THE IMPORTANCE OF SUPPRESSION AND SYNERGISM

RICHARD K. ZIMMER-FAUST,*[1] JEFFREY E. TYRE,†
WILLIAM C. MICHEL,† AND JAMES F. CASE†

**Department of Zoology, University of Queensland, St. Lucia, Brisbane 4067, Queensland, Australia and*
†Department of Biological Sciences and the Marine Science Institute
University of California Santa Barbara, California, 93106

Abstract

The California spiny lobster, *Panulirus interruptus*, failed to exhibit appetitive feeding or locomotion in response to a low molecular weight fraction (<1000 daltons) prepared from a sea water extract of muscle from abalone, a natural prey. This lack of response was caused by chemical suppressants, rather than by lack of stimulatory compounds. Excitatory responses were induced by single, low molecular weight compounds, but these responses were inhibited by suppressants which occur naturally in the muscle fraction. Amino and organic acids were found highly stimulatory to lobsters, but nucleotides and sugars were not. A mixture of monocarboxylic amino acids and dicarboxylic organic acids was much more effective in elliciting behavior than either of the constituents tested alone, at the same overall concentration. Mixtures which combined either ammonium or urea with amino or organic acids significantly reduced behavioral activity caused by these latter substances. Results indicate that tests of single chemicals cannot always reliably predict the stimulatory properties of solutions, combining even as few as two or more compounds. The stimulatory properties of complex odorants, including prey extracts, are best assessed by fractionating and then combining and testing the fractions in bioassays of factorial design.

Introduction

Behavioral investigations of feeding and electrophysiological studies of chemosensory afference have usually shown that decapod crustaceans are sensitive to low molecular weight compounds. Of these, organic nitrogenous substances and organic acids are the most stimulatory (Laverack, 1963; Case, 1964; McLeese, 1970; Kay, 1971; Shepheard, 1974; Allen *et al.*, 1975; Hindley, 1975; Ache *et al.*, 1978; Johnson and Ache 1978; Mackie *et al.*, 1980; Derby and Atema, 1982a, b). Carbohydrates (Ashby and Larimer, 1965; Hartman and Hartman, 1977; Zimmer *et al.*, 1979; Robertson *et al.*, 1981) and nucleotides (Shelton and Mackie, 1971; Carr and Thompson, 1983) also evoke responses. It is generally assumed that low molecular weight substances are the dominant natural feeding attractants for marine decapods since they stimulate receptors, cause behavioral responses, and are highly soluble and diffusable in sea water (Ache *et al.*, 1976). These latter properties are thought to result in their rapid release from tissues of prey and from carrion (Rittschof, 1980; Zimmer-Faust and Case, 1982a). Because low molecular weight compounds may provide the earliest chemosensory clues to distant food sources, it is generally assumed that decapod predators emphasize their detection in food search and feeding.

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[1] Present address: Marine Science Institute, University of California, Santa Barbara, California, 93106.

Recent electrophysiological investigations have demonstrated that certain low molecular weight substances, abundant in animal flesh, suppress the neural responses of lobster and crab antennule chemoreceptors to stimulatory compounds (Gleeson and Ache, 1983; Johnson and Atema, 1983). While this finding is interesting, there are as yet no clear behavioral correlates for these physiological observations. Suppressants may be found to serve a vital role in the control of foraging and feeding by these animals. For example, suppressants might reduce ingestion of harmful substances, reduce the search for food of low caloric value, or reduce locomotory activity of an animal in the vicinity of a valuable food item, increasing the likelihood of food discovery. Most previous behavioral studies of chemoreception in feeding and food search by decapod crustacea have focused on the role of low molecular weight substances as excitants, typically with tests performed of single chemicals and simple mixtures (McLeese, 1970; Kay, 1971; Allen *et al.,* 1975; Fuzessery and Childress, 1975; Hindley, 1975; Hamner and Hamner, 1977; Hartman and Hartman, 1977; Ache *et al.,* 1978; Robertson *et al.,* 1981; Carter and Steele, 1982; Zimmer-Faust and Case, 1982b). These experiments assumed that summative chemosensory inputs directly control behavior, and little attention has been given to CNS processing. However, in the few studies in which interactions among stimuli were considered synergy was observed (*e.g.,* Shelton and Mackie, 1971; Mackie and Shelton, 1972; Carr, 1978; Robertson *et al.,*1981).

Under field conditions, we have found the California spiny lobster, *Panulirus interruptus* (Randall), arriving in greatest numbers at abalone muscle (*Haliotis* spp.) baits, after 24–48 h (Zimmer-Faust and Case, 1982a). This occurs even though small molecules (primary amines) are released from the baits predominantly over the first 3 h. Moreover, laboratory experiments demonstrate that *P. interruptus* responds significantly to a high molecular weight fraction (>1000 daltons), but not to a low molecular weight fraction (<1000 daltons) prepared from abalone muscle (Zimmer-Faust *et al.,* 1984). Though our field and laboratory observations are in agreement, they differ from what is commonly believed true for decapods, namely, that low molecular weight substances control foraging and feeding. Our results are further at odds with previous electrophysiological investigations which show that *P. interruptus* possesses chemoreceptors sensitive to low molecular weight compounds, occurring abundantly in the flesh of abalone (Fuzessery and Childress, 1975; Lindsey, 1976).

These considerations prompted the present investigation into the behavioral responses of *Panulirus* to low molecular weight substances. Compounds were individually assayed for their ability to stimulate early arousal and appetitive phases of feeding. Some were effective, thus demonstrating specifically for *Panulirus* that inability to respond to the low molecular weight fraction of abalone does not arise from behavioral insensitivity to low molecular weight substances. It was further experimentally demonstrated that this inability was caused by chemical suppressants, and that both synergistic and suppressant interactions occur among substances naturally residing in the tissues of prey and carrion of lobsters. Our results demonstrate that the contributions made by specific chemical agents to the stimulatory capacity of a complex prey extract, cannot be properly specified without considering the entire chemosensory integrative capacity of the responding organism.

Materials and Methods

General procedures

General procedures and apparatus were identical to those previously described (Zimmer-Faust and Case, 1983; Zimmer-Faust *et al.,* 1984). Animals captured in

traps or by hand (SCUBA) at More Mesa reef, 4 km east of the UCSB campus, were brought immediately to our laboratory and held in 3000 l aquaria for 14 days before experiments were initiated. Incoming animals were tattoo marked for individual recognition (Kuris, 1971), and only hard-shelled animals of 60–68 mm carapace length were used. Animals were fed abalone muscle, mackerel muscle (*Scomber japonicus*), and opened mussels (*Mytilus californianus*) and were deprived of food for 24 h before testing.

Lobsters were individually tested for responses to chemical solutions in rectangular aquaria, 30 × 30 × 13 cm, a size permitting the control of stimulus flow, without inhibiting behavior. Sea water (980 ml/min) entered each aquarium at a velocity of ~50 cm/s, from a head-tank maintained under constant hydrostatic pressure. Stimulants were introduced from a reservoir (10 ml/7 s) by opening a three-way valve. Dilution associated with stimulus delivery was 1.02×10^{-3} ($\pm 0.13 \times 10^{-3}$ S.D.) times original concentrations, as previously determined by fluorometric measurement of fluorescein dye dilution (Zimmer-Faust and Case, 1983). Concentrations reported are not corrected for this dilution.

Lobsters were tested once in 48 h for a maximum of 6 times during a 14-day period. They were put in experimental aquaria 90–120 min prior to testing and usually settled within 30–40 min. Observations of behavior were initiated 1 min before introduction of a chemical solution and continued for 3 min afterwards. All trials were conducted according to a double-blind protocol, in which the observer was unaware of the composition of test or control solutions being tested. Order of stimulus presentation did not influence the behavior of animals, since for each substance the proportion responding was unrelated to the test sequence. All solutions were prepared from analytical grade reagents and 5 μm filtered sea water, adjusted to pH 7.8 before testing.

Antennule flicking and wiping, pereiopod probing, and locomotion were monitored, since these are behaviors commonly exhibited by *Panulirus* and other decapods in response to chemicals associated with food (*e.g.*, Maynard and Dingle, 1963; McLeese, 1970; Kay, 1971; Mackie and Shelton, 1972; Snow, 1973; Allen *et al.*, 1975; Fuzessery and Childress, 1975; Hindley, 1975; Pearson and Olla, 1977; Pearson *et al.*, 1979; Zimmer-Faust and Case, 1982b). Appetitive feeding was defined as the occurrence of increased flicking (>1.0 flick/s), wiping and probing each within a 3 min trial period. Further justification for the emphasis on these behavioral acts appears elsewhere (Zimmer-Faust *et al.*, 1984), and their definitions are given in Table I. Chemicals were considered stimulatory when proportions of responding animals differed significantly from the proportion responding to sea water ($P < 0.05$). A G-Test for Independence was used with Williams' correction for 2 × 2 contingency tables, in analyzing data from experiments presenting test solutions to differing groups of animals. A binomial test was used (Sokal and Rohlf, 1981, p. 774; $P = q = 0.5$), for experiments presenting test solutions to the same group of animals, with changes in individual responsivities compared.

Experiment 1: responses to single compounds

Previous investigations showed that lobsters are unresponsive to a low molecular weight fraction (<1000 daltons) of an extract prepared from abalone muscle (Zimmer-Faust *et al.*, 1984). For this reason, tests were conducted to determine if lack of response is caused by behavioral insensitivity to low molecular weight substances. Thirty-two compounds were individually assayed at 10^{-2} *M*, and each chemical was tested on 20 different animals in conjunction with 40 sea water controls.

TABLE I

Definitions of behavioral elements in appetitive feeding and locomotion by Panulirus

Act	Definition
Feeding	
Antennule flicking	Vertical deflection of a lateral antennular flagellum to a position nearly contacting the medial flagellum. A response was defined as >1.0 flick per second.
Leg probing	Any non-locomotor movement of a pereiopod, either raking a dactyl across the substratum, or elevating a dactyl to a position no longer in contact with the substratum.
Antennule wiping	A downward and vertical deflection of an antennule, resulting in simultaneous contact of both antennular flagella with the third maxillipeds.
Locomotion	A laterally or anteriorly directed movement of the body to a distance, >1/2 carapace length.

Experiment 2: interaction between glycine and the <1000 dalton fraction of freeze-dried abalone muscle extract (FDAME)

In the first experiment, glycine was found to be the most stimulatory of all tested substances (see Results, Table II). Because a low-molecular weight fraction of FDAME is ineffective in stimulating feeding in *Panulirus,* yet contains a high concentration of glycine (4.5×10^{-4} *M*) (Zimmer-Faust *et al.,* 1984), this finding suggested the existence of suppressants within the fraction. An experiment was conducted to test for this possibility.

A low molecular weight fraction of FDAME was prepared from a standard extract (6.00 g/l) of lyophilized abalone muscle and filtered sea water, by the procedures of Zimmer-Faust *et al.* (1984). Ultrafiltration of the extract was performed using an Amicon model 402 pressure ultrafiltration vessel and UM-2 membrane, with ultrafiltrate (<1000 daltons) collected undiluted, and stored frozen (−20°C) in aliquots. Aliquots of 10^{-4} *M* glycine, <1000 fraction with 10^{-4} *M* glycine added, and sea water were each presented to the same 27 animals. The application of glycine, by itself, served to control for the possibility that lack of response might be caused by factors other than chemical composition. It was expected that the glycine-enhanced low molecular weight fraction would be ineffective, if suppressants for glycine existed.

Experiment 3: interactions between glycine and other defined compounds

Experiments employing the <1000 dalton fraction could not be used to identify the mechanism(s) of feeding suppression, because its constituents might have two types of effects. They might bind to and thereby limit the action of stimulatory molecules, or they might act directly to influence chemosensory processes. Therefore, to approach this question we were constrained to examine the simplest of interactions in this system, namely, those between glycine and other defined compounds. Such tests could demonstrate if suppression directly involves either primary chemosensory processes or CNS mechanisms, by eliminating the possibility of chemical binding or chelating among assayed substances.

Tests with glycine, urea, and ammonium. We first explored the interaction between glycine and urea. Urea was selected because it is highly abundant in an extract known

to be noxious to lobsters (J. E. Tyre, upubl. data), prepared from the muscle of angel shark (*Squatina californica*), and because it cannot bind glycine under our present test conditions. Aliquots of 10^{-2} *M* glycine, 10^{-2} *M* glycine plus 10^{-2} *M* urea, 10^{-2} *M* urea, and sea water were each presented to the same 52 animals.

Experiments were then performed to investigate the possible interaction between glycine and ammonium. Ammonium was selected because of its close similarity to the major molecular subcomponent of urea, and because of its abundance in the low molecular weight fraction of FDAME (1.0×10^{-3} *M*). Thus, ammonium might serve as a natural suppressant in FDAME to glycine-induced feeding responses. Like urea, it does not bind to glycine under present test conditions. Tests were conducted injecting aliquots of 10^{-2} *M* glycine, 10^{-2} *M* glycine plus 10^{-2} *M* ammonium, 10^{-2} *M* ammonium, and sea water, each to the same 32 animals.

Additional tests were performed to further examine the interaction between glycine and ammonium. These tests were conducted by injecting aliquots of 10^{-3} *M* ammonium, both by itself, and in combination with 10^{-2} and 10^{-4} *M* glycine. Tests were also performed by injecting aliquots of 10^{-2} and 10^{-4} *M* glycine without ammonium added to serve as standards for comparisons with previous trials. Each solution and a sea water control were introduced to the same 20 animals.

Tests with glycine and taurine. Another series of tests were performed to investigate the possible interaction between glycine and taurine. Taurine was selected because it is the most abundant free amino acid in FDAME (6.0×10^{-3} *M*), yet is relatively ineffective in causing appetitive feeding responses (see Results, Table II). The structure of taurine differs significantly from those of urea and ammonium, and tests for interactions between taurine and glycine provided a useful comparison. Taurine does not bind glycine under present test conditions. Aliquots of 10^{-2} *M* glycine, 10^{-2} *M* glycine plus 10^{-2} *M* taurine, 10^{-2} *M* taurine, and sea water were each presented to the same 20 animals.

Experiment 4: interactions between succinic acid, urea, and ammonium

An additional experiment was performed to establish whether any observed suppression by urea and ammonium is specific to stimulations by glycine, or whether urea and ammonium might act more generally to suppress behavior caused by other non-nitrogenous compounds. Succinic acid was selected as an alternative test compound to glycine, because it evokes appetitive responses (see Results, Table II) yet does not possess an amine or amide group as do glycine, urea, and ammonium. Aliquots of 10^{-2} *M* succinic acid, 10^{-2} *M* succinic acid plus 10^{-2} *M* urea, 10^{-2} *M* succinic acid plus 10^{-2} *M* ammonium, 10^{-2} *M* urea, 10^{-2} *M* ammonium, and sea water were each presented to the same 32 animals.

Experiment 5: interactions between amino and organic acids

Thus far, only those interactions were considered that could possibly lead to a suppression of feeding. Obviously, interactions might also occur to potentiate responses. In the first experiment of this study, testing single compounds, we found at least two major groups of stimulatory molecules to be involved: (1) the small, uncharged monocarboxylic α amino acids and (2) the negatively charged, dicarboxylic organic acids (see Results, Table II). To investigate possible synergistic interactions among these substances, we performed experiments injecting mixtures of equimolar amino acids (glycine, alanine, serine), equimolar organic acids (oxalic, succinic), and equimolar amino and organic acids, each at a total molarity of 10^{-4}. Each mixture and a sea water (control) were tested on the same 20 animals.

RESULTS

Responses to low molecular weight compounds

All tested chemicals were detected, as demonstrated by increased rates of antennule flicking (Table II). However, antennule flicking was a poor indicator of overall stim-

TABLE II

Appetitive feeding and locomotor responses to single chemicals[a]

Compound[b]	Feeding component			
	Antennule flicking (Detection)	Pereiopod probing	Antennule wiping	Locomotion
Amino acids (L-isomers)				
glycine	1.00***	0.95***	0.75***	0.15
alanine	1.00***	0.75**	0.65***	0.05
serine	1.00***	0.70**	0.60***	0.15
methionine	1.00***	0.70**	0.40**	0.10
isoleucine	1.00***	0.65*	0.35*	0.05
leucine	1.00***	0.70**	0.30*	0.10
glutamic acid	1.00***	0.65*	0.30*	0.15
valine	1.00***	0.55	0.30*	0.10
threonine	1.00***	0.40	0.30*	0.00
histidine	0.95***	0.40	0.30*	0.00
lysine	1.00***	0.60*	0.25*	0.20
phenylalanine	1.00***	0.45	0.25*	0.00
aspartic acid	0.95***	0.35	0.05	0.00
arginine	0.85**	0.35	0.05	0.00
taurine	1.00***	0.70**	0.00	0.05
Organic acids (L-isomers)				
succinic	1.00***	0.65*	0.55***	0.30*
malic	1.00***	0.30	0.15	0.15
ascorbic	0.85**	0.35	0.10	0.05
citric	0.95***	0.30	0.10	0.05
oxalic	1.00***	0.60*	0.05	0.35*
propionic	0.80*	0.35	0.00	0.05
Carbohydrates (D-isomers)				
glucose	0.95***	0.65*	0.10	0.05
mannose	1.00***	0.45	0.05	0.15
fructose	0.90**	0.45	0.05	0.20
maltose	1.00***	0.40	0.00	0.10
Nucleotides (5'-monophosphates)				
cytosine (CMP)	0.95***	0.35	0.00	0.15
guanine (GMP)	0.95***	0.20	0.00	0.00
adenine (AMP)	0.90**	0.20	0.00	0.05
Miscellaneous				
betaine	0.95***	0.75**	0.35*	0.15
trimethylamine	0.90**	0.60*	0.15	0.15
glutathione (reduced)	0.85**	0.45	0.15	0.00
ammonium	1.00***	0.70**	0.10	0.00
Sea water (controls)	0.45	0.33	0.05	0.10

[a] Data are expressed as proportions of responding animals.

[b] Each compound was tested on 20 different animals at an injected concentration of 10^{-2} *M*. Sea water was tested on 40 different animals.

* The difference between the proportion of animals responding to test *versus* sea water (control) solutions is significant (G-Test and Williams' Correction: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

ulatory capacity, since the activation of feeding sequences varied tremendously among the detected substances. Of the 15 amino acids tested, only 8 induced all three of the appetitive behaviors, with glycine, alanine, and serine being the most effective (Table II). Alpha amino acids were ranked by their ability to effect appetitive feeding and the ranked order was found to be inversely correlated with molecular weight (Kendall's Tau: $\tau = -0.64$, $P < 0.01$, n = 14). Alpha amino acids having uncharged R-groups were also more effective than charged molecules in activating appetitive behavior (Mann-Whitney U Test: $T_X = 20$, $P = 0.009$, n = 9, m = 5); thus, both molecular weight and charge contributed significantly to the stimulatory capacity of these substances. Glycine initiated appetitive feeding at concentrations of 10^{-2} and 10^{-4}, but not at 3.33×10^{-5} or 10^{-6} *M* (Fig. 1). Appetitive feeding did not occur at any test concentration of taurine, though increased antennule flicking and pereiopod probing were observed at the highest concentration. Succinic acid and betaine were the only other chemicals to initiate all three of the appetitive behaviors, while sugars and nucleotides were generally ineffective as stimulants. Only two compounds, oxalic and succinic acids, caused significant locomotor responses (Table II).

Interaction between glycine and the <1000 dalton fraction of FDAME

Responses to the glycine-enhanced, low molecular weight fraction were not significantly different from those to sea water, for any assayed behavior (Table III). Significance was approached in the case of antennule flicking, showing that the en-

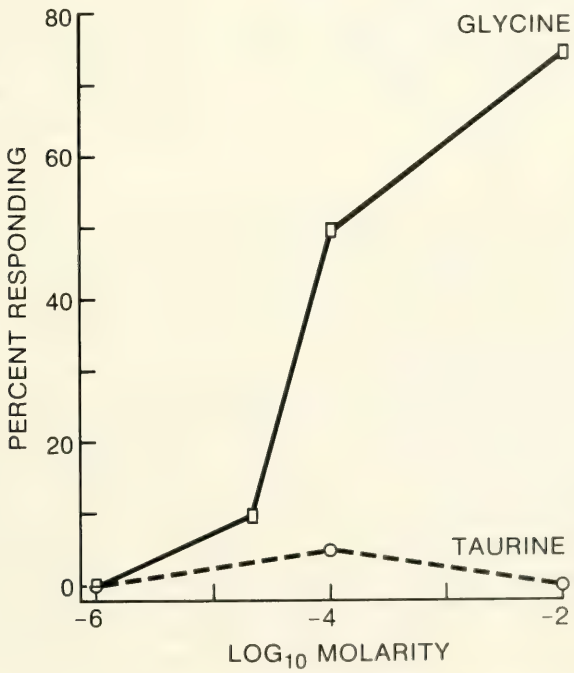

FIGURE 1. Proportions of animals showing appetitive feeding responses to glycine and to taurine. Glycine was an effective stimulant at 10^{-2} and 10^{-4} *M*, while taurine was ineffective at all tested concentrations. Five percent of all animals responded to sea water controls. Appetitive feeding was defined as the occurrence of probing, wiping and increased flicking, each within a 3-min trial period.

TABLE III

Interaction between glycine and the <1000 dalton fraction of FDAME[a]

Test solution	Injected glycine concentration (M)		Feeding component: Antennule flicking (detection)	Pereiopod probing	Antennule wiping	Locomotion
glycine		1.0×10^{-4}	0.96*	0.78**	0.48**	0.09
<1000 dalton fraction	4.5×10^{-4}	5.5×10^{-4}				
glycine	1.0×10^{-4}		0.93	0.56	0.22	0.06
sea water		——	0.78	0.44	0.15	0.06

[a] Data are expressed as proportions of responding animals, and n = 27.

* The difference between the proportion of animals responding to test *versus* sea water (control) solutions is significant (Binomial Test: $*P < 0.05$, $**P < 0.01$).

hanced fraction was probably detected by lobsters, but was otherwise ineffective as a stimulant. The 10^{-4} M glycine (control) solution caused significant responses, relative to sea water, demonstrating that response failure of the glycine-enhanced low molecular weight fraction was not caused by factors other than chemical composition. Responses to glycine were compared with those to the glycine-enhanced fraction, and significant differences were found for pereiopod probing (Binomial Test: k = 6, Y = 6, $P = 0.02$) and for antennule wiping (k = 10; Y = 9, $P = 0.01$). This clearly demonstrated an existence of suppressants in the low molecular weight fraction, since the enhanced fraction contained 5.5× more glycine stimulant than the control (10^{-4} M) solution. These results are even more impressive when it is considered that glycine was only one of several known stimulatory compounds in the low molecular weight fraction, which includes alanine and glutamic acid among others (Zimmer-Faust *et al.*, 1984).

Interaction between glycine, urea, and ammonium

Both urea and ammonium acted as mild stimulants when tested at 10^{-2} M, inducing flicking and probing but not wiping or locomotion (Table IV, Expts. A and B). Glycine evoked all four assayed behaviors at 10^{-2} M, but did not initiate locomotor responses at 10^{-4} M. Responses to glycine differed significantly from those to urea-glycine, in pereiopod probing (Binomial Test: $P < 0.005$), antennule wiping ($P < 0.01$) and locomotion ($P = 0.01$), which showed that urea was inhibitory to the glycine response (Table IV, Expt. A). Glycine-induced wiping responses were inhibited by ammonium, when ammonium (10^{-2} and 10^{-3} M) was combined with either a high (10^{-2} M) or low (10^{-4} M) concentration of glycine (Table IV, Expts. B and C and Binomial test: $P < 0.01$, all comparisons). Ammonium was also inhibitory to probing and to locomotion when combined at 10^{-2} or 10^{-3} M with a high concentration (10^{-2} M) of glycine ($P < 0.05$, all comparisons). Results identified at least one inhibitory interaction (ammonium-glycine) that could affect the stimulatory capacity of FDAME. It is of major interest that both urea and ammonium were by themselves slightly stimulatory, since this means that not all combinations of substances which are stimulants when presented singly necessarily heighten behavioral responses.

Interaction between glycine and taurine

Proportions of animals responding to glycine and to taurine were almost identical to those of previous experiments (Table IV, Experiment D). Glycine evoked significant

TABLE IV

Interactions between glycine and other defined compounds[a]

Experiment	Test solution	Injected concentration		Feeding component: Antennule flicking (detection)	Pereiopod probing	Antennule wiping	Locomotion
A	glycine		1.0×10^{-2}	1.00***	0.77***	0.63***	0.21*
(n = 52)	glycine + urea	1.0×10^{-2} + 1.0×10^{-2}	2.0×10^{-2}	1.00***	0.58**	0.36*	0.08
	urea		1.0×10^{-2}	1.00***	0.48**	0.04	0.08
	sea water		——	0.35	0.21	0.04	0.04
B	glycine		1.0×10^{-2}	1.00***	0.78***	0.66***	0.22*
(n = 32)	glycine + ammonium	1.0×10^{-2} + 1.0×10^{-2}	2.0×10^{-2}	1.00***	0.47**	0.34*	0.06
	ammonium		1.0×10^{-2}	1.00***	0.56**	0.00	0.06
	sea water		——	0.34	0.19	0.06	0.06
C	glycine		1.0×10^{-2}	1.00***	0.95***	0.80***	0.20
(n = 20)			1.0×10^{-4}	1.00***	0.75***	0.55***	0.05
	glycine + ammonium	1.0×10^{-2} + 1.0×10^{-3}	1.1×10^{-2}	1.00***	0.75***	0.40**	0.00
		1.0×10^{-4} + 1.0×10^{-3}	1.1×10^{-3}	1.00***	0.85***	0.20	0.00
	ammonium		1.0×10^{-3}	1.00***	0.55	0.00	0.00
	sea water		——	0.40	0.35	0.05	0.05
D	glycine		1.0×10^{-2}	1.00***	0.75***	0.70***	0.20
(n = 20)	glycine + taurine	1.0×10^{-2} + 1.0×10^{-2}	2.0×10^{-2}	1.00***	0.80***	0.65***	0.20
	taurine		1.0×10^{-2}	1.00***	0.70***	0.00	0.10
	sea water		——	0.45	0.40	0.05	0.10

[a] Data are expressed as proportions of responding animals.

* The difference between the proportion of animals responding to test *versus* sea water (control) solutions is significant (Binomial Test: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

flicking, probing, and wiping responses, but not locomotion. Non-significant changes in locomotor behavior resulted more from a slightly elevated response to sea water (controls) and from a smaller sample size, than from a reduced responsivity to glycine. Taurine significantly induced flicking and probing, but not wiping or locomotion. Responses to the taurine-glycine solution were exhibited by nearly equal proportions of animals, as were those to the glycine (control) solution, and no significant differences were found (Table IV, Expt. D). This showed that taurine has no effect on stimulation by glycine, which is important, since it clearly demonstrates that suppression is dependent on the nature of the interacting compounds, in our test system.

Interaction between succinic acid, urea, and ammonium

Urea and ammonium evoked flicking and probing, while succinic acid induced each of the four assayed behaviors (Table V). Comparisons of responses to succinic acid and to interactive solutions (urea-succinic acid and ammonium-succinic acid) identified significant differences in antennule wiping (Binomial Test: $P \leq 0.03$, both

TABLE V

Interactions between succinic acid and other defined compounds[a]

Test solution	Injected concentration (M)		Antennule flicking (detection)	Pereiopod probing	Antennule wiping	Locomotion
succinic acid		1.0×10^{-2}	0.97***	0.63**	0.38***	0.28**
succinic acid + urea	1.0×10^{-2} + 1.0×10^{-2}	2.0×10^{-2}	0.94***	0.50**	0.16	0.06
succinic acid + ammonium	1.0×10^{-2} + 1.0×10^{-2}	2.0×10^{-2}	1.00***	0.50**	0.06	0.13
urea		1.0×10^{-2}	1.00***	0.53**	0.06	0.09
ammonium		1.0×10^{-2}	1.00***	0.56**	0.00	0.06
sea water		—	0.34	0.19	0.06	0.06

[a] Data are expressed as proportions of responding animals, and n = 32.

* The difference between the proportion of animals responding to test *versus* sea water (control) solutions is significant (Binomial Test: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$).

comparisons) and locomotion ($P \leq 0.03$, both comparisons). Thus, both urea and ammonium are inhibitory to succinic acid, as well as to glycine.

Interaction between amino and organic acids

Amino and organic acids were more effective when tested as combined stimuli, than when tested singly. This was demonstrated by our finding that the mixture of amino acids and the mixture of organic acids were only slightly stimulatory, at the tested concentration (10^{-4} M), while a mixture combining these substances was highly effective (Table VI). Data showed that an increase in responsivity by test animals

TABLE VI

Interaction between amino and organic acids[a]

Test solution	Injected concentration (M)		Feeding Component: Antennule flicking (detection)	Pereiopod probing	Antennule wiping	Locomotion
glycine + alanine + serine	3.33×10^{-5} + 3.33×10^{-5} + 3.33×10^{-5}	1.00×10^{-4}	0.95***	0.40	0.10	0.15
succinic acid + oxalic acid	5.00×10^{-5} + 5.00×10^{-5}	1.00×10^{-4}	0.75*	0.30	0.00	0.05
glycine + alanine + serine + succinic acid + oxalic acid	2.00×10^{-5} + 2.00×10^{-5} + 2.00×10^{-5} + 2.00×10^{-5} + 2.00×10^{-5}	1.00×10^{-4}	0.90**	0.45	0.40***	0.30*
sea water (controls)		——	0.40	0.35	0.00	0.00

[a] Data are expressed as proportions of responding animals, and n = 20.

* The difference between the proportion of animals responding to test *versus* sea water (control) solutions is significant (Binomial Test: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$).

was the result of differences in the qualitative chemical composition of mixtures, because overall chemical concentration was maintained constant. Proportions of animals initiating wiping and locomotion were actually greater in response to the combined amino-organic acid mixture, than those in response to the amino acid mixture added with those in response to the organic acid mixture. The interaction between amino and organic acids was clearly synergistic.

Discussion

Our results show that low molecular weight compounds induce both appetitive feeding and locomotion in the California spiny lobster, *Panulirus interruptus*. This is noteworthy, because it differs from our previous findings with *P. interruptus*, in which a low molecular weight fraction (<1000 daltons) of abalone muscle was unable to stimulate appetitive feeding in laboratory tests (Zimmer-Faust *et al.*, 1984), and also failed to attract lobsters to baited traps in field experiments (Zimmer-Faust and Case, 1982a). Apparent disparity in our findings is not the result of differences in experimental procedures, since these were the same for all laboratory tests, nor is it the result of a lack of stimulatory substances within the low molecular weight fraction. Several compounds occurring in the fraction were found to be stimulatory when presented individually to *P. interruptus*. The failure of the low molecular weight fraction must, therefore, be attributed to a presence of substances that either affect the chemical senses of lobsters, or bind to, and thereby limit the action of, stimulatory molecules. In distinguishing between these possibilities, we first performed an experiment to directly demonstrate that feeding suppression is caused by chemicals residing in the low molecular weight fraction of abalone. Suppression was then determined to affect the chemosensory processes of lobsters in experiments showing ammonium and urea inhibiting the behavior caused by glycine and succinic acid, without binding these substances.

Synergism in the stimulation of lobsters was observed upon combining monocarboxylic amino acids, including glycine, with dicarboxylic organic acids, including succinic acid. Synergism is generally recognized to arise, at least in part, from the simultaneous stimulation of different chemoreceptor sites, each varying in chemical specificity (*e.g.*, Shelton and Mackie, 1971; Mackie and Shelton, 1972; Dethier, 1976; Kroeze, 1981). Our results suggest that glycine and succinic acid may be stimulating different receptor sites and possibly different cell populations. Because both urea and ammonium were inhibitory to glycine and to succinic acid, it is further suggested that (1) urea and ammonium may act non-specifically to suppress behavior caused by afferences from at least two different receptor sites, and (2) competitive interactions between suppressants (urea and ammonium) and stimulants seem unlikely to occur, at both receptor sites for glycine and for succinic acid, due to major differences in the molecular structures and charges of these substances.

Neurophysiological experiments are now needed to test these hypotheses. Data are available from at least one neurophysiological study of chemoreceptors on the antennules of *Panulirus interruptus*, and these generally support our findings. In this investigation, two classes of chemoreceptors were identified. One responded predominantly to mono-, and the other responded to di-carboxylic amino acids (Fuzessery and Childress, 1975). It was postulated in that study that through stimulation of receptors having different specificities, a mixture of mono- and di-carboxylic amino acids might heighten feeding responses, presumably through a CNS mechanism. Results of present behavioral experiments are very similar to those postulated, though we did substitute dicarboxylic organic acids for their amino acid counterparts.

It is well known that suppressants occur in the tissues of a variety of plants and

animals, to be used in chemical defenses against predation (*e.g.*, see Sondheimer and Simeone, 1970; Chapman, 1974; Kittredge *et al.*, 1974; Shorey and McKelvey, 1977; Faulkner and Ghiselin, 1983 for reviews). In the present study, however, we observed abalone muscle to contain suppressants, yet it is readily consumed by lobsters. Furthermore, abalone muscle causes appetitive feeding responses in lobsters (Zimmer-Faust *et al.*, 1984), and it is highly attractive to lobsters when used as a bait (Zimmer-Faust and Case, 1982a). This demonstrates that suppressants are not in this circumstance operating strictly in chemical defense. We consider an alternative, namely, that sensitivities to suppressants are employed by lobsters to enhance discriminatory abilities. It has been clearly demonstrated for both insects and for vertebrates, that sensitivities to stimulants and to suppressants gives greater control over the modulation and tuning of feeding, according to overall quality of a chemical mixture or food odor (*e.g.*, Dethier, 1966, 1976; Fishman, 1971; Shumake *et al.*, 1971; Harborne, 1982a, b; Thompson *et al.*, 1983).

The paradox that lobsters evidently should invoke and also over-ride suppressant sensitivities, as in the case of abalone muscle, is easily resolved by considering that suppressants may be widespread. They may simply occur either in greater abundances or in an absence of stimulants in tissues of non-preferred foods. Our findings with ammonium and urea support such a conclusion. Each is a major product of nitrogen catabolism and is widespread in tissues of marine invertebrates (Campbell, 1973). Strict avoidance of these substances is impossible by feeding lobsters, though we have found lobsters avoiding the cephalothoraces of crustacean prey and carrion, tissues which accumulate and excrete ammonium and urea. Ammonium is produced in copious amounts by both anaerobic and aerobic decomposing bacteria (*e.g.*, Kjosbakken *et al.*, 1983), and it is non-nutritive to decapod crustacea. Avoidance and reduced ingestion of this compound would seem beneficial, particularly if not associated with ingestion of high quality, nutritive substances.

It must be questioned why ammonium and urea suppress stimulation by other compounds, yet are slightly stimulatory by themselves. This is best explained by the finding that antennule flicking (Snow, 1973; Pearson and Olla, 1977; Price and Ache, 1977; Pearson *et al.*, 1979; Schmitt and Ache, 1979; present study) and pereiopod movements (Atema and Engstrom, 1971) are in part associated with a generalized alerting response of decapods to chemical substances, and it is these behaviors that are evoked by ammonium and urea. Flicking and probing contrast sharply to antennule wiping, a behavior exhibited by *P. interruptus* predominantly in response to food-related chemical stimuli (J. E. Tyre, unpubl. data), and not induced by either ammonium or urea. It has been suggested by previous investigators that ammonium acts as a major feeding stimulant to the American lobster, *Homarus americanus* (Carter and Steele, 1982; Derby and Atema, 1982a). While this may be true, we point out that ammonium is released by invertebrate and vertebrate *predators* of lobsters, by bacterial decomposers, and by lobsters themselves, as well as by invertebrate prey species.

Our identification of suppressants raises several interesting questions concerning experimental methods in investigations of crustacean chemoreceptive and feeding behavior. In particular, studies which rely on tests of single compounds may not properly define the natural responsivities of test animals. This is suggested by our finding that single, low molecular weight substances, such as glycine, initiate behavioral responses when presented by themselves, but do not always contribute to the stimulatory capacity of complex, prey extracts. Furthermore, chemicals that are slightly stimulatory by themselves (*e.g.*, ammonium and urea) can profoundly reduce the stimulatory capacity of even simple mixtures.

Further difficulties arise upon considering investigations based on extracts of natural tissues and component fractions. These studies may incorrectly specify the potencies of component fractions, unless each fraction is tested both by itself and in all possible combinations. This is because potency is often non-additive, and the stimulatory capacity of a specific compound or fraction cannot always be predicted from those of others. For example, we have found FDAME to contain glycine at 5.0×10^{-4} *M* and to induce appetitive feeding in 40% of all tested animals (Zimmer-Faust *et al.*, 1984). A five-fold dilution of glycine (1.0×10^{-4} *M*), tested by itself, was found to induce appetitive feeding in a slightly higher proportion of animals (50%). It might be concluded incorrectly from these results that glycine alone is responsible for all activity generated by whole extract (FDAME). This we know is untrue, since the <1000 dalton fraction of FDAME is unstimulatory.

Our results for *Panulirus* indicate that, if compounds and fractions are to be properly identified for their contributions to the stimulatory capacity of prey odors, then experiments must be performed to assess the interactions which occur among the stimuli. This can best be accomplished by using bioassays in which fractions are prepared and tested both by themselves and in combination, in experiments of either factorial or combinatorial design. This way, interactions between chemical components can be identified for their influences on behavior by analyzing the responses of test animals in a multiway analysis of variance, where each fraction is treated as an independent factor (Sokal and Rohlf, 1981), or by comparing the deviation of response values from those predicted by derivations of simple, additive and non-additive models. The exact nature of each interaction can then be described according to its synergistic or additive properties, and according to its facilitative or inhibitory effect on behavior.

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LITERATURE CITED

ACHE, B. W., FUZESSERY, AND W. E. S. CARR. 1976. Antennular chemosensitivity in the spiny lobster, *Panulirus argus:* Comparative tests of high and low molecular weight stimulants. *Biol. Bull.* **151**: 273–282.

ACHE, B. W., B. R. JOHNSON, AND E. CLARK. 1978. Chemical attractants of the Florida spiny lobster, *Panulirus argus. Fla. Sea Grant Tech. Paper, No. 10.* 28 pp.

ALLEN, W. V., E. C. FREDERICK, AND R. WONG. 1975. Experiments on the development of an artificial bait for the dungeness crab, *Cancer magister* (Dana), *Sea Grant Publ. No. S.G.-7.* Humboldt State University. 25 pp.

ASHBY, E. A., AND J. L. LARIMER. 1965. Modification of cardiac and respiratory rhythms in crayfish following carbohydrate chemoreception. *J. Cell. Comp. Physiol.* **65**: 373–380.

ATEMA, J., AND D. G. ENGSTROM. 1971. Sex pheromone in the lobster, *Homarus americanus. Nature* **232**: 261–263.

CAMPBELL, J. W. 1973. Nitrogen excretion. Pp. 279–316 in *Comparative Animal Physiology, Vol. I: Environmental Physiology,* C. L. Prosser, ed. W. B. Saunders Co., Philadelphia.

CARR, W. E. S. 1978. Chemoreception in the shrimp, *Palaemonetes pugio:* The role of amino acids and betaine in elicitation of a feeding response by extracts. *Comp. Biochem. Physiol.* **61**A: 127–131.

CARR, W. E. S., AND H. W. THOMPSON. 1983. Adenosine 5′-monophosphate, an internal regulatory agent, is a potent chemoattractant for a marine shrimp. *J. Comp. Physiol.* **153:** 47–53.

CARTER, J. A., AND D. H. STEELE. 1982. Attraction to and selection of prey by immature lobsters (*Homarus americanus*). *Can. J. Zool.* **60:** 326–336.

CASE, J. 1964. Properties of the dactyl chemoreceptors of *Cancer antennarius* Stimpson and *C. productus* Randall. *Biol. Bull.* **127:** 428–446.

CHAPMAN, R. F. 1974. The chemical inhibition of feeding by phytophagous insects: a review. *Bull. Entomol. Res.* **64:** 339–363.

DERBY, C. D., AND J. ATEMA. 1982a. Chemosensitivity of walking legs of the lobster *Homarus americanus:* Neurophysiological response spectrum and thresholds. *J. Exp. Biol.* **98:** 303–315.

DERBY, C. D., AND J. ATEMA. 1982b. Narrow-spectrum chemoreceptor cells in the walking legs of the lobster *Homarus americanus:* Taste specialists. *J. Comp. Physiol.* **146:** 181–189.

DETHIER, V. G. 1966. Feeding behaviour. Pp. 46–58 in *Insect Behaviour,* P. T. Haskel, ed. *R. Entomol. Soc. Lond. Symp. No. 3.*

DETHIER, V. G. 1976. *The Hungry Fly: A Physiological Study of the Behavior Associated with Feeding.* Harvard Univ. Press, Cambridge. 489 pp.

FAULKNER, D. J., AND M. T. GHISELIN. 1983. Chemical defense and evolutionary ecology of dorid nudibranchs and other opisthobranch gastropods. *Mar. Ecol. Prog. Ser.* **13:** 295–301.

FISHMAN, I. Y. 1971. Taste responses in the red fox. *Physiol. Zool.* **44:** 171–176.

FUZESSERY, Z. M., AND J. J. CHILDRESS. 1975. Comparative chemosensitivity to amino acids and their role in the feeding behavior of bathypelagic and littoral crustaceans. *Biol. Bull.* **148:** 522–538.

GLEESON, R. A., AND B. W. ACHE. 1983. Mixture suppression of primary chemoreceptor responses: neurophysiological evidence in taurine sensitive cells. *Soc. Neurosci. Abstr.* **9:** 1024.

HAMNER, P., AND W. HAMNER. 1977. Chemosensory tracking of scent trails by the planktonic shrimp *Acetes sibogae australis. Science* **195:** 886–888.

HARBORNE, J. B. 1982a. Insect feeding preferences. Pp. 121–154 in *Introduction to Ecological Biochemistry.* Academic Press, New York.

HARBORNE, J. B. 1982b. Feeding preferences of vertebrates including man. Pp. 155–177 in *Introduction to Ecological Biochemistry.* Academic Press, New York.

HARTMAN, H. B., AND M. S. HARTMAN. 1977. The stimulation of filter feeding in the porcelain crab, *Petrolisthes cinctipes* (Randall) by amino acids and sugars. *Comp. Biochem. Physiol.* **56A:** 19–22.

HINDLEY, J. P. R. 1975. The detection, location and recognition of food by juvenile banana prawns, *Penaeus merguiensis* de man. *Mar. Behav. Physiol.* **3:** 193–210.

JOHNSON, B. R., AND B. W. ACHE. 1978. Antennular chemosensitivity in the spiny lobster, *P. argus:* Amino acids as feeding stimulants. *Mar. Behav. Physiol.* **5:** 145–157.

JOHNSON, B. R., AND J. ATEMA. 1983. Narrow spectrum chemoreceptor cells in the antennules of the American lobster. *Neurosci. Lett.* **41:** 145–150.

KAY, M. 1971. Isoleucine: An inducer of the feeding response in decapod crustaceans. *Experientia* **27:** 103.

KITTREDGE, J. S., F. T. TAKAHASHI, J. LINDSEY, AND R. LASKER. 1974. Chemical signals in the sea: marine allelochemics and evolution. *Fish. Bull.* **72:** 1–11.

KJOSBAKKEN, J., I. STORRO, AND H. LARSEN. 1983. Bacteria decomposing amino acids in bulk-stored Capelin (*Mallotus villosus*). *Can. J. Fish. Aquat. Sci.* **40:** 2092–2097.

KROEZE, J. H. A. 1981. Reduced sweetness and saltiness judgements of NaCl-sucrose mixtures depend on a central inhibitory mechanism. Pp. 161–174 in *Determination of Behavior by Chemical Stimuli,* J. E. Steiner, ed. I. R. L. Press Ltd., London.

KURIS, A. M. 1971. Population interactions between a shore crab and two symbionts. Ph.D. Dissertation, University of California, Berkeley.

LAVERACK, M. S. 1963. Aspects of chemoreception in crustacea. *Comp. Biochem. Physiol.* **8:** 141–151.

LINDSEY, J. E. 1976. Contact chemoreceptor mechanisms in the California rock lobster, *Panulirus interruptus* (Randall). Ph.D. Dissertation, University of California, Santa Barbara.

MACKIE, A. M. 1973. The chemical basis of food detection in the lobster *Homarus gammarus. Mar. Biol.* **15:** 103–108.

MACKIE, A. M., P. T. GRANT, R. G. J. SHELTON, B. T. HEPPER, AND P. R. WALNE. 1980. The relative efficiencies of natural and artificial baits for the lobster, *Homarus gammarus:* Laboratory and field trials. *J. Cons. Int. Exp. Mer.* **39:** 123–129.

MACKIE, A. M., AND R. G. J. SHELTON. 1972. A whole-animal bioassay for the determination of food attractants of the lobster *Homarus gammarus. Mar. Biol.* **14:** 217–221.

MAYNARD, D. M., AND H. DINGLE. 1963. An effect of eyestalk ablation on antennular function in the spiny lobster, *Panulirus argus. Z. Vgl. Physiol.* **46:** 515–540.

MCLEESE, D. W. 1970. Detection of dissolved substances by the American lobster (*Homarus americanus*) and olfactory attraction between lobsters. *J. Fish. Res. Board Can.* **27:** 1371–1378.

PEARSON, W. H., AND B. L. OLLA. 1977. Chemoreception in the blue crab (*Callinectes sapidus*). *Biol. Bull.* **153:** 346–354.

PEARSON, W. H., P. C. SUGARMAN, D. L. WOODRUFF, AND B. L. OLLA. 1979. Thresholds for detection and feeding behavior in the dungeness crab, *Cancer magister*. *J. Exp. Mar. Biol. Ecol.* **39:** 65–78.

PRICE, R. B., AND B. W. ACHE. 1977. Peripheral modification of chemosensory information in the spiny lobster. *Comp. Biochem. Physiol.* **57A:** 249–253.

RITTSCHOF, D. 1980. Chemical attraction of hermit crabs and other attendants to simulated gastropod predation sites. *J. Chem. Ecol.* **6:** 103–118.

ROBERTSON, J. R., J. A. FUDGE, AND G. K. VERMEER. 1981. Chemical and live feeding stimulants of the sand fiddler crab, *Uca pugilator* (Bosc). *J. Exp. Mar. Biol. Ecol.* **53:** 47–64.

SCHMITT, B. C., AND B. W. ACHE. 1979. Olfaction: responses of a decapod crustacean are enhanced by flicking. *Science* **205:** 204–206.

SHELTON, R. G. J., AND A. M. MACKIE. 1971. Studies on the chemical preferences of the shore crab, *Carcinus maenas* (L.). *J. Exp. Mar. Biol. Ecol.* **7:** 41–49.

SHEPHEARD, P. 1974. Chemoreception in the antennule of the lobster, *Homarus americanus*. *Mar. Behav. Physiol.* **2:** 261–273.

SHOREY, H. H., AND J. J. MCKELVEY, eds. 1977. *Chemical Ecology of Insect Behavior: Theory and Application*. John Wiley and Sons, New York. 414 pp.

SHUMAKE, S. A., R. D. THOMPSON, AND C. J. CAUDILL, 1971. Taste preference behavior of laboratory versus wild Norway rats. *J. Comp. Physiol. Psychology* **77:** 489–494.

SNOW, P. J. 1973. The antennular activities of the hermit crab, *Pagurus alaskensis* (Benedict). *J. Exp. Biol.* **58:** 745–766.

SOKAL, R. R., AND F. J. ROHLF. 1981. *Biometry*. W. H. Freeman and Co., San Francisco. 859 pp.

SONDHEIMER, E., AND J. B. SIMEONE, eds. 1970. *Chemical Ecology*. Academic Press, Inc., New York. 336 pp.

THOMPSON, R. D., D. J. ELIAS, S. A. SHUMAKE, AND S. E. GADDIS. 1983. Taste preference of the common vampire bat (*Desmodus rotundus*). *J. Chem. Ecol.* **8:** 715–721.

ZIMMER, R. K., D. P. COOK, AND J. F. CASE. 1979. Chemosensory induced bradycardia in the kelp crab, *Pugettia producta* (Randall). *J. Exp. Mar. Biol. Ecol.* **38:** 135–150.

ZIMMER-FAUST, R. K., AND J. F. CASE. 1982a. Odors influencing foraging behavior of the California spiny lobster, *Panulirus interruptus*, and other decapod crustacea. *Mar. Behav. Physiol.* **9:** 35–58.

ZIMMER-FAUST, R. K., AND J. F. CASE. 1982b. Organization of food search in the kelp crab, *Pugettia producta* (Randall). *J. Exp. Mar. Biol. Ecol.* **57:** 237–255.

ZIMMER-FAUST, R. K., AND J. F. CASE. 1983. A proposed dual role of odor in foraging by the California spiny lobster, *Panulirus interruptus* (Randall). *Biol. Bull.* **164:** 341–353.

ZIMMER-FAUST, R. K., W. C. MICHEL, J. E. TYRE, AND J. F. CASE. 1984. Chemical induction of feeding in California spiny lobster, *Panulirus interruptus* (Randall): Responses to molecular weight fractions of abalone. *J. Chem. Ecol.* **10:** 957–971.

Reference: *Biol. Bull.* **167**: 354–370. (October, 1984)

REPRODUCTION, EMBRYONIC ENERGETICS, AND THE MATERNAL-FETAL RELATIONSHIP IN THE VIVIPAROUS GENUS *SEBASTES* (PISCES: SCORPAENIDAE)

GEORGE W. BOEHLERT* AND MARY M. YOKLAVICH

College of Oceanography and Marine Science Center, Oregon State University, Newport, Oregon 97365

ABSTRACT

Reproduction in the scorpaenid genus *Sebastes* has been characterized as primitive ovoviviparity. In the black rockfish, *S. melanops,* egg size is small (0.8 mm), but the gestation period is 37 days and larvae at birth are well developed, with a remnant of yolk and the ability to initiate feeding. To test the hypothesis that this species is viviparous with additional maternal nutrition, we studied embryonic energetics and morphology. Catabolism during development utilized 64% of the yolk energy, resulting in a maximum yolk utilization efficiency of 36%, similar to oviparous fishes. Calorimetry, however, demonstrates that 81% of the initial yolk energy is present at birth. Thus approximately 70% of the catabolic energy is contributed by the maternal system during gestation. Microscopic analysis of embryonic epidermis suggests no specializations for nutrient uptake. Histological observations, however, reveal that the hindgut is functional approximately 22–25 days after fertilization. Thus, we suggest that nutrition occurs through consumption and assimilation of ovarian fluid.

Reproductive modes in the Scorpaenidae have apparently evolved from simple oviparity in seven of eight subfamilies, to lecithotrophic viviparity in more primitive members of the subfamily Sebastinae, through matrotrophic viviparity in *Sebastes.* This pattern involved progressively longer retention of embryos until after organogenesis and functional differentiation of the gut, facilitating this rather primitive form of embryonic nutrition among matrotrophic viviparous species.

INTRODUCTION

Reproductive modes in the teleost fishes historically have been grouped into the categories oviparity, ovoviviparity, and viviparity. Oviparous species shed gametes from the body and fertilization and embryonic development are external. In ovoviviparity and viviparity [referred to herein as lecithotrophic and matrotrophic viviparity, respectively, after Wourms (1981)], fertilization is internal and development proceeds within the female reproductive system. The evolution of these reproductive modes involved a compromise between high reproductive rates with low survival and low reproductive rates with high survival. The former is exemplified in the oviparous fishes, the latter in viviparous fishes, although variations exist within each mode of reproduction. The traditional differences between lecithotrophic and matrotrophic viviparity involve the provision of maternal nutrition after oogenesis; it is lacking in strictly lecithotrophic viviparous species. There is a continuum of levels of maternal nutrition in matrotrophic viviparous fishes, but in most species the actual maternal-fetal relationships during gestation are unknown (Wourms, 1981). The coelacanth

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* Present address: Southwest Fisheries Center Honolulu Laboratory, National Marine Fisheries Service, NOAA, Honolulu, Hawaii 96812.

Latimeria, for example, was described as a lecithotrophic viviparous species by Smith *et al.* (1975), but was later shown to be a matrotrophe despite a lack of specialized structures for nutrient uptake (Wourms *et al.,* 1980). Further research on the maternal-fetal relationship will help us understand the evolution of viviparity in fishes (Wourms, 1981).

In the family Scorpaenidae, live-bearing is present in the subfamily Sebastinae. The most advanced genus, *Sebastes,* has a center of distribution in the North Pacific and is comprised of some 106 species worldwide (Barsukov, 1981). Reproduction in this genus is considered to be primitive among viviparous species, with no maternal nutrition (Wourms and Cohen, 1975; Wourms, 1981). Fecundity approaches that of the most highly fecund oviparous species, with individual fecundity to 2,300,000 in *S. paucispinis* (Phillips, 1964) and weight specific fecundity reaching 500 eggs per gram body weight (MacGregor, 1970; Boehlert *et al.,* 1982). Egg sizes range from about 0.7 to 1.5 mm, the gestation period is approximately 1–2 months long, and larval size at birth is relatively small, ranging from 4 to 9 mm (Fujita, 1957; Moser *et al.,* 1977). The larvae are relatively well developed, however, and are generally born at a developmental stage with organogenesis complete, jaws developed, and the ability to initiate feeding. Moser and Butler (1981), in an attempt to rear larvae of *S. dalli,* suggested that the lack of success in rearing *Sebastes* larvae may be related to the timing of birth; larvae in the wild are well developed at birth whereas those used in laboratory rearing are typically born prematurely. They speculated that additional maternal nutrition might be critical to complete organogenesis late in gestation. To test the hypothesis that reproduction in *Sebastes* is matrotrophic viviparity, we describe the energetics and nutrition of embryonic black rockfish, *S. melanops,* during gestation.

Materials and Methods

Experimental animals

Sebastes melanops is a fairly shallow-living rockfish, generally inhabiting waters from 8 to 55 m (Barsukov, 1981); adults inhabit nearshore rocky reefs from Baja California to the Aleutian Islands (Hart, 1973). All females are mature at an age of 9 years and a length of about 43 cm off Oregon (McClure, 1982), and give birth predominantly during January through March (Laroche and Richardson, 1980). Larvae are pelagic, as is typical of *Sebastes* (Ahlstrom, 1961), and emigrate to nearshore, intertidal, and estuarine areas at ages from 4 to 6 months (Boehlert and Yoklavich, 1983).

Adult *S. melanops* were collected on 8–9 January 1982 by hook and line from a sport fishing vessel off Seal Rock, Oregon in 10–20 m of water. Excess swim bladder gas was removed with hypodermic needles. Fish were held in large circular tanks (1.2 m diameter, 700-liter capacity) supplied with circulating sea water at 10°C (±1°C) during experiments. Following a 2-week period of adjustment to laboratory conditions, sex and stage of ovarian development were determined by catheterization and gentle suction; fish were tagged and segregated on the basis of reproductive state. Of the 16 females, 8 were gravid with embryos at various stages of development, 3 were preovulatory, and 5 were either immature or spent. The females with developing embryos were individually isolated in small circular tanks (0.7 m diameter, 120-liter capacity). Over time, despite separation from males, the three preovulatory females ovulated. The ova were fertilized, and subsequently underwent embryonic development. This is evidence of sperm storage since, in many species of *Sebastes,* insemination occurs up to six months prior to fertilization (Sorokin, 1961; Moser, 1967a).

Developing embryos were obtained by extracting ovarian samples from the 11 ripe females on a weekly basis from 22 January 1982 until parturition. Adult fish were anesthetized with tricaine methanesulfonate (MS-222; 75 mg/liter sea water) and catheterized using a sterile glass pipette. Oppenheimer's (1937) classification of developmental stages of *Fundulus heteroclitus* and Moser's (1967a) description of *S. paucispinis* development were used to identify stages of *S. melanops* embryos. Following Moser (1967a) and Shimizu and Yamada (1980), Oppenheimer's (1937) stages through stage 32 were found adequate for description of *Sebastes* development. Samples from different females from unfertilized eggs to extruded larvae, were taken for estimates of dry weight, ash, carbon and nitrogen, and for measurements of oxygen consumption. Examination of egg diameter from six different females revealed little variation, with mean 0.83 mm (standard deviation 0.026) and range 0.80–0.87 mm.

Calorimetry, nitrogen, and protein analysis

The maternal nutritional contribution to embryonic development was analyzed using bioenergetics and nitrogen and protein budgets. Calorimetry was used to estimate the energy content of developing embryos at eight stages, from unfertilized eggs to extruded larvae. After catheterization of gestating embryos, dry weights were measured to the nearest μg on a Perkin-Elmer AD-2Z microbalance. Groups of embryos were dried to a constant weight at 60°C. Ash content was determined after 3 hours combustion in a muffle furnace at 500°C. Samples were analyzed for carbon and nitrogen content using a Perkin-Elmer model 240-B carbon-hydrogen-nitrogen analyzer. The nitrogen-corrected equation from Salonen *et al.* (1976) was used to convert percent carbon to calories as follows:

$$\text{cal/embryo} = ([0.65\ (\%C) - 12.237]239)\ W$$

where C is carbon content per ash-free dry weight (AFDW) of sample and W is the AFDW of the embryo. While this method is based upon invertebrates, the estimated energy content of unfertilized yolk (6281 cal/g AFDW) is within the ranges of caloric estimates for marine fish eggs as summarized by Robertson (1974). Further, this relationship appears to hold for a diverse group of taxa, including phytoplankton and marine and freshwater protozoa (Finlay and Uhlig, 1981).

From additional specimens collected in 1984, protein content was determined for four developmental stages using the microbiuret method of Itzhaki and Gill (1964). Bovine serum albumin was used as a protein standard. The same specimens were analyzed for CHN content for comparison with 1982 specimens and to determine the percentage of protein nitrogen. This latter value was determined by dividing protein by 6.25.

Oxygen consumption

We measured respiration rates to compare the energy content of the developing embryos with the catabolic energy required during gestation. Embryos were removed by catheterization and placed in physiological saline (Forster and Hong, 1958) isosmotic with ovarian fluid (*ca.* 325 mOsM/kg, determined in a Wescor vapor pressure osmometer) in 15 ml respiration flasks. Experiments were performed in darkness at 10°C in a Gilson differential respirometer following standard techniques (Umbreit *et al.*, 1972). Embryos in respirometers were allowed to equilibrate for 0.5–1 hour prior to initial readings; subsequent readings were taken every 1–2 hours for 5–10 hours. Most experiments were performed with three replicates. Numbers and dry

weights of embryos per vessel were determined upon completion of experiments and results were expressed as μl O_2/embryo/h and μl O_2/mg AFDW/h.

One of the problems in determining the total embryonic oxygen consumption during gestation is that *Sebastes* is viviparous; it is therefore difficult to establish a zero time for fertilization. Length of time spent at each stage of development was determined from samples of embryos taken by successively catheterizing females during gestation, at approximately weekly intervals. By determining the change in stage over the interval, we were able to estimate the duration of each Oppenheimer stage at 10°C. Using these estimates, we integrated the time spent at all stages to calculate a gestation period and also to estimate the total oxygen consumption during the gestation period. We felt that this estimate would be based upon the maximum number of females and would minimize individual variability.

Morphology

Histology of the alimentary tract and the surface structure of the epidermis were examined to determine the site of energy uptake in the developing embryos. For histological examination, small groups of catheterized embryos were preserved in isosmotic buffered 2% glutaraldehyde. Embryos were imbedded in acrylic resin (LR White), sectioned at 2 μm, and stained in hematoxylin and methylene blue-basic fuchsin after Bennett *et al.* (1976). The epidermis was examined with an AMR 1000 scanning electron microscope after fixation and preparation following Dobbs' (1974) methodology.

RESULTS

Stage duration and length of gestation

Length of time spent at each stage of development was determined from samples of embryos which were taken at intervals throughout gestation. Twenty-two samples from 8 fish provided 11 estimates to establish the relation between stage duration (D), measured in days/stage, and stage (S), which presented the midpoint of development stage between two sample intervals:

$$D = 0.0452S^{1.090}, \qquad n = 11, \qquad r^2 = 0.89$$

This relationship was nearly linear, with stage duration increasing with increasing stage number (Fig. 1). From the model of stage duration, a cumulative age of the embryo (time since fertilization) was calculated for stages 1 to 32 and plotted as a function of Oppenheimer developmental stage (Fig. 1). From stage 32 (estimated from the model to be reached in 31.2 days) to parturition, an average of 5.8 days elapsed for the 11 females in the study. The average gestation period at 10°C is therefore estimated to be 37 days. This agrees closely with our estimates from gestation periods of three individual fish which ovulated and were fertilized in the laboratory ($n = 3$, $x = 36.3$ days, $S = 0.58$).

Oxygen consumption rates

Embryonic *S. melanops* representing seven stages of development from eight females were used to determine total oxygen consumed during gestation. Depending upon stage of development, between 24 and 229 embryos were placed in each 15 ml respirometer flask. Embryos were judged to be inactive in respirometer flasks, but we could not observe activity during shaking of the flasks. Oxygen consumption

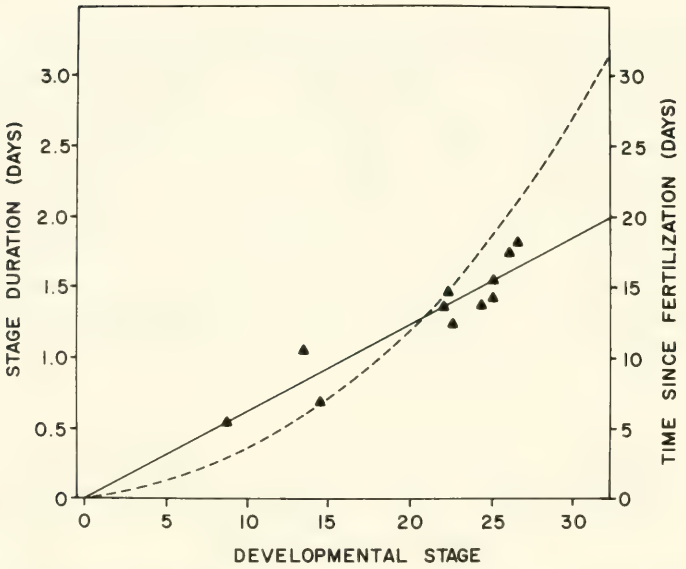

FIGURE 1. Duration of developmental stages during gestation of embryonic *Sebastes melanops* at 10°C. Developmental stages are determined according to Oppenheimer (1937). Stage durations (solid line) were estimated by successive catheterization of gestating females. The time since fertilization (dashed line) is an integration of the curve for stage duration; we estimate approximately 32 days to stage 32 and an additional 5 days until birth.

(μl/embryo/h) increased as embryonic development progressed (Fig. 2). The lowest oxygen uptake, averaging 0.0078 O_2/embryo/h, was measured from embryos in the earliest stage of development (stage 9 and approximately 2.4 days after fertilization as estimated from the stage duration model). The oldest embryos (stage 30 and 27.4 days after fertilization) utilized an average of 0.087 μl O_2/embryo/h. Oxygen consumption (Q, μl O_2/embryo/h), expressed as a function of time since fertilization (t, in days), is described by the equation:

$$Q = 0.00321t^{1.0026}, \qquad n = 8, \qquad r^2 = 0.98 \qquad \text{(Fig. 2)}.$$

From this curve a cumulative estimate of oxygen required over the estimated gestation period of 37 days was determined to be 54.667 μl O_2/embryo. Using an oxycaloric equivalent of 0.005 cal/μl O_2 (Lasker, 1962), the catabolic component of energy used during gestation would correspond to 0.273 calorie.

Energy and protein changes during development

Measurements of weight and caloric content for developing embryos are presented in Table I. Eight stages are represented, including unfertilized eggs just prior to ovulation and the extruded larvae. In general, both weight and caloric content gradually decreased throughout development. The AFDW of an unfertilized egg was 67.5 μg. From the CHN analysis, percent carbon and nitrogen were determined and caloric content was estimated to be 0.429 calorie/egg. A correction was made for caloric contribution by the chorion to total available energy, since the chorion provides no energy to the embryo. The average chorion AFDW of 1.11 μg was converted to a caloric value of 0.005 calorie, assuming 50% carbon content. The available energy to the embryo is

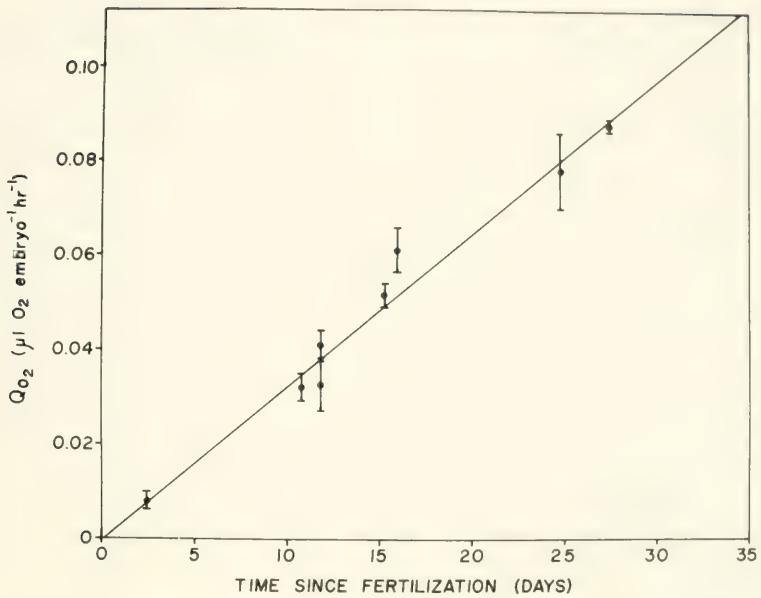

FIGURE 2. Oxygen consumption (μl O_2 embryo^{-1} h^{-1}) for embryonic *Sebastes melanops* as a function of time since fertilization at 10°C. Each point represents the mean of two to three observations on embryos from the same female; scale bars represent ±2 S.E.

therefore 0.424 calorie. At hatching, when the relatively well-developed larva still has a remnant of yolk, caloric measurements suggest that 81% of the initial egg energy remains in yolk and embryonic tissue. Nitrogen increases as a percentage of embryo dry weight with increasing time since fertilization, but this is balanced by decreasing total weight such that total nitrogen remains static (Table I; n = 12, r = −0.23, $P > 0.10$). Carbon, on the other hand, actually decreases with development (Table I; n = 12, r = −0.92, $P < 0.01$).

TABLE I

Weight, carbon, nitrogen, and caloric values of Sebastes melanops *embryos throughout gestation*

Oppenheimer stage	Dry weight embryo (μg)	AFDW embryo (μg)	Nitrogen (μg)	Carbon (μg)	C/N	Caloric content of embryo (cal/embryo)	Caloric content (embryo-chorion)
Nonfertilized egg	71	67.5	7.0	40.2	5.74	0.429	0.424
13	74	70.3	7.4	41.2	5.57	0.435	0.430
16	70	66.2	7.4	38.6	5.22	0.407	0.402
18	70	65.8	7.3	37.7	5.16	0.394	0.389
22	66	61.1	7.1	35.0	4.93	0.365	0.360
28	71	65.3	6.9	35.8	5.19	0.365	0.360
31	70	63.9	7.2	33.3	4.63	0.330	0.325
Larvae at birth	66.7	60.4	7.0	33.6	4.80	0.345	0.345

Developmental stages are determined after Oppenheimer (1937). (C/N = carbon to nitrogen ratio; AFDW = ash-free dry weight.)

If nonprotein nitrogen remained constant over development, total protein would remain static. In the 1984 examples, however, although the nitrogen remained static, protein decreased significantly during development ($n = 24$, $r = -0.73$, $P < 0.01$; Table II). These data suggest that nonprotein nitrogen increases from 1% in the earliest embryos studied to 27% in newly extruded larvae. This seems unusual in light of other values for teleosts, which range from 9 to 18% of total nitrogen (Niimi, 1972), and may be indicative of a nitrogenous source of maternal nutrition.

Comparing calorimetry and catabolic energy utilization provides evidence that additional maternal nutrition is provided during gestation. Based upon the calculation of 0.273 catabolic caloric necessary during gestation, 64.3% of the initial energy is used for catabolism. This suggests that the efficiency of yolk utilization from fertilization to parturition is approximately 36%. Calorimetry, however, shows that 81% of the initial energy investment remains at parturition. The difference represents energy from the maternal system. It is probable that the nutritional substance is nitrogenous, such as amino acids or peptides, since the total nitrogen remains nearly constant during gestation (Tables I, II). Comparing the time course of gestation and the energy content remaining as calculated by catabolism and calorimetry reveals the approximate times and developmental stages at which the nutrition is provided (Fig. 3). Energy content remaining after catabolism continued to decrease over development; measurements from calorimetry are similar through an embryonic age of about 20–25 days, corresponding to Oppenheimer stage 26.

The location of uptake of nutritional substances by embryonic *S. melanops* was approached from a structural standpoint, with examination of the epidermal surfaces and the histology of the alimentary tract. Epidermal uptake has been suggested for certain species and has been documented in the clinid *Clinus superciliosus* by Veith (1980). In embryonic *S. melanops,* we examined epidermal tissues at Oppenheimer stages 21, 31, and in larvae shortly after parturition, representing approximately 13, 29, and 38 days post-fertilization, respectively. At stage 21, prior to provision of maternal nutrients (Fig. 3), the epidermis on the nape (posterior to the cranium) and yolk sac regions is relatively smooth, with only cell boundaries distinct. Small, 3–4 μm depressions are apparent irregularly over the epidermis; these depressions may represent the locations of subdermal sacciform cells (Bullock, 1980). Only weak evidence of microridges, and no microvilli, are present (Fig. 4a). Epidermal microridges on stage 31 embryos are better developed in both nape (Fig. 4b) and yolk sac regions (Fig. 4c). In both of these developmental stages, relative development of the microridges decreases towards the tail. Finally in larvae shortly after birth, the microridges remain but are less distinct (Fig. 4d).

TABLE II

Comparison of protein, nitrogen, and carbon content from embryonic Sebastes melanops

Oppenheimer stage	Nitrogen (μg)	Carbon (μg)	Protein (μg)	PN/TN
9	7.30 (—)	41.64 (—)	45.27 (1.20)	0.99
23	7.78 (0.26)	40.71 (1.18)	46.27 (3.97)	0.95
28	7.64 (0.04)	38.48 (0.16)	36.23 (1.53)	0.76
Larvae at birth	7.74 (0.06)	34.42 (0.09)	35.31 (3.80)	0.73

Protein was determined using the methods of Itzhaki and Gill (1964) and carbon-nitrogen by CHN analyzer. Numbers in parentheses indicate one standard deviation. (PN/TN = ratio of protein nitrogen to total nitrogen.)

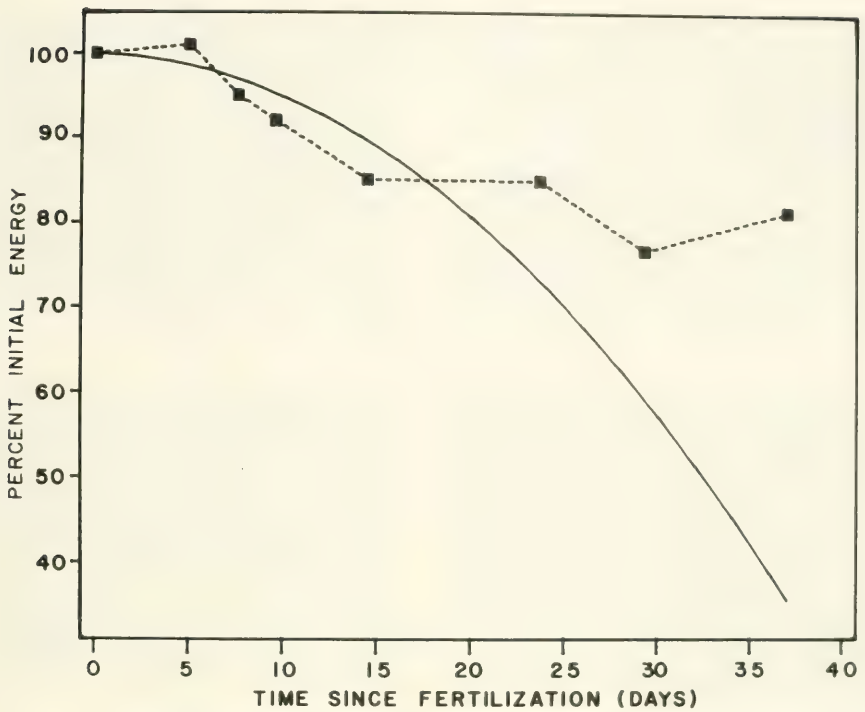

FIGURE 3. Comparison of energy budgets in embryonic *Sebastes melanops* during gestation based upon direct and indirect calorimetry. The dashed line represents caloric values of embryos at various times during gestation. The solid line represents energy utilization based upon the model of oxygen consumption during gestation and assumes an oxycalorific equivalent of 0.005 cal $(\mu l\ O_2)^{-1}$.

Histological observations on the gut of embryonic *S. melanops* demonstrate a change with increasing development. In early embryos, the foregut is not open, and the gut epithelium is relatively narrow and basophilic, with material lacking in the gut lumen (Fig. 5a). With development past stage 27, however, observation of whole embryos and serial sections show that the gut is open. Few histological changes are noted in either foregut or midgut regions, with the obvious exception of acidophilic material in the lumen (Fig. 5b). In the hindgut or rectum, however, the gut lumen also contains an amorphous substance and the height of the hindgut epithelium is greater. Additionally, there are large, supranuclear inclusions and some vacuolation (Fig. 5c). Finally, at stage 31, the hindgut epithelium is greatly expanded with marked increases of acidophilic supranuclear inclusions (Fig. 5d).

DISCUSSION

The reproductive mode in *Sebastes* has previously been termed "primitive" ovoviviparity (Amoroso, 1960; Wourms and Bayne, 1973; Wourms, 1981) despite a lack of experimental studies on this genus. The high fecundity, lack of structural modifications for embryonic nutrition, and small embryonic and larval size suggested that embryos received no nutrition beyond oogenesis. Our approach in this study has followed several studies on yolk utilization in oviparous species, and therefore comparisons of energy utilization are relevant here. Values useful for such comparisons

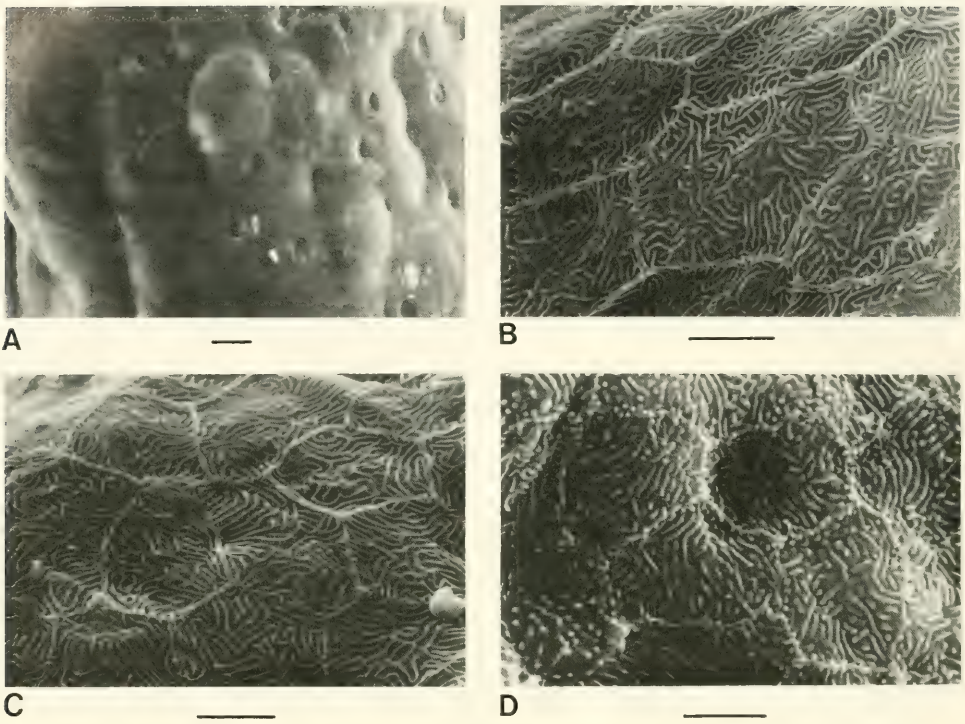

FIGURE 4. Scanning electron micrographs of the epidermis of embryonic and larval *Sebastes melanops*. a. Stage 21 embryo (13 days post-fertilization), nape region. Note the 3–4 μm depressions. Although the cell boundaries are distinct, the microridges are only poorly developed. b. Stage 31 embryo (29 days post-fertilization), nape region. c. Stage 31 embryo, yolk sac region. d. Larva shortly after parturition, nape region. Scale bars indicate 10 μm.

are the "plastic efficiency coefficient" (PEC) of Gray (1928) and the "apparent energetic efficiency" (AEE) of Needham (1931). The former is the ratio of dry weight of the developed embryo to that of yolk at fertilization, and the latter is the ratio of the caloric values of the same stages. Value of PEC and AEE from oviparous teleosts range from 0.24 to 0.65 and 0.26 to 0.79, respectively, and clearly demonstrate the expected decrease in mass and energy content during development (Table III). Among viviparous species, Thibault and Schultz (1978) noted values of PEC from 0.61 to 18.83 within the genus *Poeciliopsis;* their data suggest that *P. monacha* is a lecithotrophe, whereas the other three species receive varying levels of additional maternal nutrition during gestation.

Development in *Sebastes* shows some similarities to that in oviparous species. Oxygen consumption continually increases during gestation (Fig. 2), but the length of gestation is much greater than incubation time of equivalent sized eggs from oviparous species. Ware (1975), for example, compiled information on incubation times, sizes, and temperatures of marine species with pelagic eggs and demonstrated a highly significant relationship of egg diameter and incubation time. This relationship predicts an incubation time of 1.3 days given the egg size of *S. melanops* or an egg size of 4.4 mm given its incubation time; this is in marked contrast to the 37-day incubation and 0.8 mm egg size. The pattern of energy utilization described above also demonstrates important differences. The only prior information on *Sebastes,*

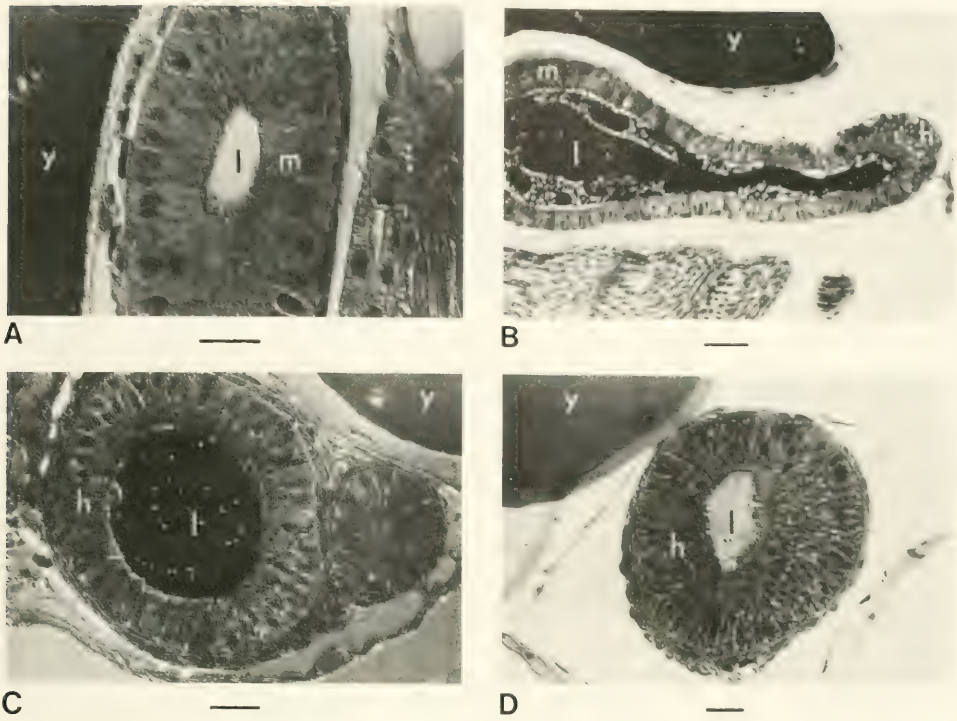

FIGURE 5. Histological sections of gut tissue in various stages of embryonic *Sebastes melanops*. a. Stage 21 embryo (13 days post-fertilization), midgut region dorsal to the yolk sac. Note the columnar gut epithelium and empty lumen (scale bar = 20 μm). b. Longitudinal section of the midgut-hindgut region of a stage 28 embryo (24 days post-fertilization). Note the densely staining, acidophilic substance throughout the lumen of the gut (scale bar = 50 μm). c. Cross section of the hindgut of a stage 28 embryo. Note the expanded hindgut epithelium with material in the lumen (scale bar = 20 μm). d. Cross section of the hindgut of a stage 31 embryo (29 days post-fertilization). The lumen contains an amorphous, granular substance, and the epithelial cells are characterized by supranuclear granules (scale bar = 20 μm). (h = hindgut epithelium; l = lumen; m = midgut epithelium; y = yolk sac.)

that of Hsaio (unpubl.), however, supports the idea of lecithotrophic viviparity in *S. marinus*. He demonstrated ". . . a pronounced fall in the dry weight of the developing egg, indicating little or no maternal contribution" (Needham, 1942). The only accessible data of Hsaio, however, are presented in Scrimshaw (1945), where the dry weight of the newly fertilized egg is presented as 0.003 g. This value is more than an order of magnitude greater than dry weights observed in the present study (Table I), and in Moser (1967a), which are in the range of 70–100 g. While the egg size in *S. marinus* is slightly larger than that of some Pacific species (Taning, 1961), it is doubtful that it could be 30-fold greater in dry weight. We therefore consider his unpublished values unreliable. Our value for PEC, 0.90, could conceivably be at the upper range of oviparous or lecithotrophic viviparous species (Table III). Furthermore, the value of AEE determined by oxgyen consumption is within the range of values determined for oviparous species. Relative to other species where both values are available, however, the relationship of the two values is inconsistent with the interpretation of lecithotrophic viviparity. Calorimetry demonstrates that the caloric content of the larva at parturition represents 81% of the initial energy invested at oogenesis. This value is more representative of that expected given the value of 0.90 for PEC. The sum of energy used

TABLE III

Efficiency of yolk utilization in selected fishes

	Temperature °C	PEC	AEE	Source
Oviparous species				
Tautoga onitis	16	0.41	0.36	Laurence, 1973
	19	0.29	0.26	Laurence, 1973
	22	0.30	0.26	Laurence, 1973
Congiopodus leucopaecilus	11.5	—	0.64	Robertson, 1974
Solea solea	10	—	0.68	Fluchter and Pandian, 1968
	15	—	0.55	Fluchter and Pandian, 1968
	20	—	0.47	Fluchter and Pandian, 1968
Limanda ferruginea	4–12	0.24–0.45	0.32–0.41	Howell, 1980
Paralichthys dentatus	16	0.61	0.62	Johns and Howell, 1980
Sardinops caerulea	14	0.48	0.79[1]	Lasker, 1962
Viviparous species				
Poeciliopsis monacha	22–32	0.61	—	Thibault and Schultz, 1978
P. lucida	22–32	1.34	—	Thibault and Schultz, 1978
P. prolifica	22–32	3.50	—	Thibault and Schultz, 1978
P. turneri	22–32	18.83	—	Thibault and Schultz, 1978
Sebastes marinus	—	0.66	—	Hsiao, unpub. (cited in Schrimshaw, 1945)
S. melanops	10	0.90	0.81 (0.36)[1]	Present study

PEC refers to the "plastic efficiency coefficient" (Gray, 1928) which is the ratio of dry weight of the developed embryo to that of the fertilized egg. AEE refers to the "apparent energetic efficiency" of Needham (1931), which is the same ratio using caloric values rather than dry weight. Values for viviparous species refer to embryos at parturition, when a small amount of yolk may remain.

[1] Utilization is based upon oxygen consumption and oxycalorific equivalents.

for catabolism (0.273 calorie) and that remaining at birth (0.345 calorie) represents 1.46 times that initially available at fertilization. A comparison of energy utilization during embryogenesis (Fig. 3) suggests that energy is first provided between Oppenheimer stages 22 and 28, corresponding to approximately 15 and 24 days after fertilization, respectively.

Viviparous teleosts display several adaptations for embryonic nutrition; the range of variation has been discussed in reviews on viviparity in fishes (Amoroso, 1960; Wourms, 1981). The most notable adapations for nutrient exchange are generally present in the embryonic, rather than maternal tissues. In some species, transfer of materials and respiratory gases may simply take place across the epithelium of the body and fins (Veith, 1979, 1980). In the embiotocids, the median fins become spatulate and highly vascularized, serving the function of respiratory gas exchange (Webb and Brett, 1972; Dobbs, 1975). Trophotaeniae are extensions of gut tissue present in several viviparous families which serve an absorptive function (Turner, 1937; Amoroso, 1960; Wourms and Cohen, 1975). These structures are continuous with the gut and assimilate ovarian secretions or histotrophe; similarly, other species may simply ingest ovarian fluid without externally specialized trophotaeniae (Dobbs, 1975; Veith, 1980).

In *S. melanops* embryos there are no notably specialized structures for uptake of histotrophe equivalent to those of more advanced viviparous species (Wourms and Bayne, 1973; Wourms, 1981). The epidermal structure of *S. melanops* embryos (Fig. 4) suggests that uptake does not occur there. Although there are numerous microridges

which generally characterize teleost epidermis (Yamada, 1968; Depeche, 1973; Roberts *et al.*, 1973), microvilli usually characterize the absorptive surfaces on embryos of viviparous fishes, both on epidermal (Veith, 1980) and trophotaenial surfaces (Lombardi and Wourms, 1978). Veith (1980), for example, noted numerous epidermal microvilli situated on macroridges in early embryos of *Clinus superciliosus* and demonstrated uptake of labeled compounds via autoradiography. Uptake was limited to relatively small embryos, however, as the microvilli fused to form epidermal microridges later in gestation when the gut assumed the function of nutrient uptake. In *S. melanops,* the increased development of microridges with time after fertilization (Fig. 4) may be a response to increased oxygen consumption (Fig. 2), even after parturition, since larval fish epidermis often serves a respiratory function (Weihs, 1980).

The other possible site of nutrient uptake is the gut. Examination of gross morphology of embryos during gestation demonstrates that the mouth is open and the gut continuous at Oppenheimer stage 27, approximately 22 days post-fertilization. This time correlates well with the timing of maternal nutrition as estimated in Figure 3. Histological examination of the gut agrees with this assessment (Fig. 5). In early stage embryos, the gut epithelium is relatively narrow and basophilic (Fig. 5a), a condition similar to that described by Govoni (1980) for nonfeeding yolk sac larvae of *Leiostomus xanthurus.* With development past stage 27 in embryonic *S. melanops,* however, an amorphous acidophilic material is present in the lumen of the hindgut, evidence of ingestion of histotrophe (Fig. 5b, d); this may be similar to the material observed in the expanded hindgut of stage 28 *S. schlegeli* by Shimizu and Yamada (1980). This material is absent in earlier stages. Also the most marked change in the gut epithelium occurs in the hindgut, where the epithelium is characterized by marked expansion and development of supranuclear acidophilic inclusions and vacuolation (Fig. 5d). O'Connell (1976), in diagnosing the feeding condition of larval *Engraulis mordax,* used similar supranuclear, eosinophilic inclusion bodies in the hindgut to evaluate the relative nutritional state of larvae. Actively feeding larvae were characterized by "massive" supranuclear inclusions in the hindgut; his histological figures of this state are similar to those of later stage embryos of *S. melanops.*

Evidence for uptake of exogenous substances in the hindgut supports the idea that the substance is possibly amino acids, peptides, or proteins. Iwai (1969) suggested that protein is absorbed in the hindgut of larval fishes, largely by pinocytosis. This has been confirmed by Watanabe (1981, 1982), who described the uptake and intracellular digestion of horseradish peroxidase in the guts of larvae of several teleosts. The nitrogen content of developing embryos of *S. melanops* remains nearly constant (Table I) while protein decreases (Table II). In development of most oviparous teleost larvae, protein and nitrogen decrease during development. Rogers and Westin (1981) noted a decrease of 48% in total nitrogen in developing striped bass larvae and a marked increase in the carbon:nitrogen ratio, which contrasts with the decreasing ratio in *S. melanops* (Tables I, II). Similarly, Hayes (1949) suggested that 40% of the original yolk proteins in the trout egg are used as energy sources in development. Thus while other sources of nutrition cannot be ruled out, protein or other nitrogenous energy sources are clearly involved in additional maternal nutrition of embryos.

Although the basic reproductive pattern in the genus *Sebastes* is similar among species, variations in reproductive strategy and life history pattern exist. Variations in maximum size (and therefore fecundity), age at maturity, seasonality of spawning, multiple spawning, and longevity are well documented within the genus; furthermore, intraspecific variations may occur with latitude (Boehlert and Kappenman, 1980). Small species such as *Sebastes jordani* and *S. emphaeus* may mature at age 2, reach a maximum size of 179 mm (*S. emphaeus*) and maximum ages of about 7 years,

and achieve maximum fecundity of approximately 50,000 developing embryos (Phillips, 1964; Moulton, 1975). Larger species such as *S. paucispinis* may mature at age 4 or later, reach maximum lengths of 870 mm, bear an annual or biannual brood of up to 2,300,000 developing embryos, and reach maximum ages of 30 years (Phillips, 1964; Moser, 1967a). Other investigators have documented ages greater than 80 years (Bennett *et al.*, 1982). Other species have not been studied relative to additional maternal contribution. The opaque substance in the enlarged rectum of *S. schlegeli* embryos noted by Shimizu and Yamada (1980), however, suggests a similar nutritional mode during gestation. Ultrastructural analysis of rectal cells of embryos in this species reveals that they are characterized by supranuclear inclusions similar to those noticed in *S. melanops* (Fig. 5d), also indicative of exogenous nutrition (M. Shimizu, pers. comm.). Thus the pattern of nutrition documented for *S. melanops* may occur in other species in this genus. Given the wide variety of over 100 species of *Sebastes* (Barsukov, 1981), however, there may be variability in maternal nutrition as is apparent within genera of other viviparous groups such as the poeciliids (Table III; Thibault and Schultz, 1978).

Viviparity in *Sebastes* is a significant adaptation to environmental conditions, as in other viviparous species (Amoroso, 1960; Wourms, 1981). Although Turner (1947) could draw no ecological correlations with reproductive type among the poeciliids, Thibault and Schultz (1978) suggested that the diverse reproductive mechanisms relate closely to conditions of predators, food availability, and physical environment. In marine fishes, most mortality is concentrated in the early life history during so-called "critical periods" (Hjort, 1914; Marr, 1956). In the Pacific sardine, for example, mortality may be 30% per day during the pelagic egg stage (Smith, 1973) and 10% per day during the larval stage (Lenarz, 1973). Thus brooding of embryos during development may significantly decrease early mortality due to predation and starvation. Further, less energy is necessary for protection of the embryo. In oviparous species, the chorion represent 15–33% of egg dry weight (Blaxter and Hempel, 1966; Robertson, 1974); in *S. melanops,* however, it represents only 1.6%. The protective function served by the chorion in oviparous species (Davenport *et al.*, 1981) is apparently unnecessary in the benign ovarian environment. The chorion in *S. paucispinis* embryos is only 1 μm thick and "porous" in nature (Moser, 1967b). It is probable that this chorion does not prevent movement of macromolecules, since exogenous nutrition of embryonic *S. melanops* occurs some 8 days prior to hatching from the chorion.

Within the family Scorpaenidae, seven of eight subfamilies are oviparous; only the Sebastinae is characterized by internal fertilization and viviparity. *Sebastes* is clearly the most advanced genus within this group, which also includes the genera *Hozukius* and *Helicolenus.* While the reproductive pattern of the deep-living *Hozukius* is unknown (Barsukov, 1981), *Helicolenus* is thought to be the most primitive (Krefft, 1961). While internally fertilized, *Helicolenus* lays gelatinous egg masses similar to those of the oviparous genera *Scorpaena* and *Sebastolobus;* larvae at parturition are in varying stages of development (Krefft, 1961; Graham, 1939). In the genus *Sebastiscus* (considered a subgenus of *Sebastes* by Barsukov and Chen, 1978) parturition occurs immediately after hatching in the ovary (Tsukahara, 1962). There has been some controversy concerning the stage of development at birth in *Sebastes,* but observations of natural birth generally support hatching within the ovary (Moser 1967b; Kusakari, 1978; present study). We estimate that 5 days elapse between hatching and birth for *S. melanops* at 10°C.

While *Sebastes* is the most reproductively advanced genus within the Scorpaenidae, it is generally considered primitive among viviparous fishes (Amoroso, 1960; Wourms, 1981). A primitive character present in the *Sebastes* female reproductive system is

the presence of paired ovaries which are uncommon among viviparous species (Amoroso, 1960). A unique specialization in the reproductive system is the dual arterial system supplying fresh arterial blood to each ovary, which no doubt presents a relatiely high respiratory oxygen demand on the maternal system during embryogenesis (Moser, 1967b). It is interesting to note that the genera of the subfamily Sebastinae show morphological and osteological evidence for primitive and advanced characters which correspond to the degree of reproductive specialization; *Helicolenus* is most primitive and closest to the ancestral, oviparous scorpaenines, *Sebastes* is the most advanced (Krefft, 1961; Moser and Ahlstrom, 1978).

Kusakari *et al.* (1977) observed that the larvae of *S. schlegeli* at spawning are embedded in a gelatinous matrix which gradually dissolves after pectoral fanning by the female. Such a gelatinous substance is consistent with the gelatinous egg masses of *Helicolenus, Scorpaena,* and *Sebastolobus.* Wourms (1981) questioned whether this jelly in *Helicolenus* might play some trophic function. We have not observed spawning or such a viscous matrix in *S. melanops;* thus ovarian fluid may be a more fluid histrophe in this species. This histotrophe may be produced in the granulosa cells noted by Moser (1967b) to hypertrophy in *S. paucispinis* ovaries. A second source of histotrophe may arise from resorbed embryos which die during development. Boehlert *et al.* (1982) observed degenerating embryos in fertilized *S. entomelas* ovaries and noted decreased fecundity with later developmental stages in this species. In the latter case, if embryonic death occurs, the energy contained in these embryos may be recovered by brood mates.

Reproductive similarities among genera in the subfamily Sebastinae and relationships with other genera in the family suggest that evolution of viviparity proceeded through progressively longer retention of developing embryos. In the ancestor of extant sebastines, internal fertilization was probably followed by rapid deposition of fertilized embryos (Barsukov, 1981), a condition termed ovi-ovoviviparity by Balon (1975). In *Helicolenus* retention time of fertilized embryos may be variable (Krefft, 1961), with eggs deposited shortly after fertilization, or retained for longer periods (Graham, 1939). *Sebastes* larvae, on the other hand, are typically ready to feed at birth, with organogenesis complete. Thus evolution of viviparity in the scorpaenids involved progressively greater commitments of parental care; additional nutrition from ovarian fluid, or histotrophe, may have simply developed as an "opportunistic and passive strategy" (Wourms, 1981). It would thus be interesting to investigate the range of variability in viviparity for the genus *Sebastes.*

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LITERATURE CITED

AHLSTROM, E. H. 1961. Distribution and relative abundance of rockfish (*Sebastodes* spp.) larvae off California and Baja California. *Rapp. P. V. Reun. Cons. Perm. Int. Explor. Mer* **150:** 169–176.

AMOROSO, E. C. 1960. Viviparity in fishes. *Symp. Zool. Soc. Lond.* **1:** 153–181.

BALON, E. K. 1975. Reproductive guilds of fishes: A proposal and definition. *J. Fish. Res. Board Can.* **32:** 821–864.

BARSUKOV, V. V. 1981. A brief review of the subfamily Sebastinae. *J. Ichthyol.* **21:** 1–26.

BARSUKOV, V. V., AND L. C. CHEN. 1978. Review of the subgenus *Sebastiscus* (*Sebastes, Scorpaenidae*) with a description of a new species. *Vopr. Ikhtiol.* **18:** 195–210.

BENNETT, H. S., A. D. WYRICK, S. W. LEE, AND J. H. MCNEIL, JR. 1976. Science and art in preparing tissues embedded in plastic for light microscopy, with special reference to glycol methacrylate, glass knives, and simple stains. *Stain Technol.* **51:** 79–97.

BENNETT, J. G., G. W. BOEHLERT, AND K. K. TUREKIAN. 1982. Confirmation of longevity in *Sebastes diploproa* (Pisces: Scorpaenidae) using $^{210}PB/^{226}Ra$ measurements in otoliths. *Mar. Biol.* **71:** 209–215.

BLAXTER, J. H. S., AND G. HEMPEL. 1966. Utilization of yolk by herring larvae. *J. Mar. Biol. Assoc. U. K.* **46:** 219–234.

BOEHLERT, G. W., W. H. BARSS, AND P. B. LAMBERSON. 1982. Fecundity of the widow rockfish, *Sebastes entomelas,* off the coast of Oregon. *Fish. Bull. (U.S.)* **80:** 881–884.

BOEHLERT, G. W., AND R. F. KAPPENMAN. 1980. Variation of growth with latitude in two species of rockfish (*Sebastes pinniger* and *S. diploproa*) from the northeast Pacific Ocean. *Mar. Ecol. Prog. Ser.* **3:** 1–10.

BOEHLERT, G. W., AND M. M. YOKLAVICH. 1983. Effects of temperature, ration, and fish size on growth of juvenile black rockfish, *Sebastes melanops. Env. Biol. Fish.* **8:** 17–28.

BULLOCK, A. M. 1980. A comparative study of the epidermal morphology of fishes of the order anacanthinic with reference to their ecology and distribution. *Oceanogr. Mar. Biol. Annu. Rev.* **18:** 251–315.

DAVENPORT, J., S. LONNING, AND E. KJORSVIK. 1981. Osmotic and structural changes during early development of eggs and larvae of the cod, *Gadus morhua.* L. *J. Fish Biol.* **19:** 317–331.

DEPECHE, J. 1973. Intrastructure supervicielle de al vesicule vitellaire et du sac pericardique de l'embryon de *Poecilia reticulata* (Poisson Teleosteen). *Z. Zellforsch. Mitrosk. Anat.* **141:** 235–253.

DOBBS, G. H. 1974. Soft tissues of marine teleosts. Pp. 72–82 in *Principles and Techniques of Scanning Electron Microscopy,* M. Hayat, ed. Van Nostrand Reinhold Co., New York.

DOBBS, G. H. 1975. Scanning electron microscopy of intraovarian embyros of the viviparous teleost *Micrometrus minimus* (Gibbons), (Perciformes: Embiotocidae). *J. Fish Biol.* **7:** 209–214.

FINLAY, B. J., AND G. UHLIG. 1981. Calorific and carbon values of marine and freshwater protozoa. *Helgol. Wiss. Meeresunters.* **34:** 401–412.

FLUCHTER, J., AND T. J. PANDIAN. 1968. Rate and efficiency of yolk utilization in developing eggs of the sole *Solea solea. Helgol. Wiss. Meeresunters.* **18:** 53–60.

FORSTER, R. P., AND S. K. HONG. 1958. *In vitro* transport of dyes by isolated renal tubules of the flounder as disclosed by direct visualization: Intracellular accumulation and transcellular movement. *J. Cell. Comp. Physiol.* **51:** 259–272.

FUJITA, S. 1957. On the larval stages of a scorpaenid fish, *Sebastodes pachycephalus nigricans* (Schmidt). *Jpn. J. Ichthyol.* **6:** 91–93.

GOVONI, J. J. 1980. Morphological, histological, and functional aspects of alimentary canal and associated organ development in larval *Leiostomus xanthurus. Rev. Can. Biol.* **39:** 69–80.

GRAHAM, D. H. 1939. Breeding habits of the fishes of Otago Harbor and adjacent seas. *Trans. Proc. R. Soc. N. Z.* **69:** 361–372.

GRAY, J. 1928. The growth of fish. II. The growth rate of the embryo of *Salmo fario. J. Exp. Biol.* **6:** 110–124.

HART, J. L. 1973. Pacific fishes of Canada. *Fish. Res. Board Can. Bull.* **180:** 740 pp.

HAYES, F. R. 1949. The growth, general chemistry, and temperature relations of salmonid eggs. *Q. Rev. Biol.* **24:** 281–308.

HJORT, J. 1914. Fluctuations in the great fisheries of northern Europe viewed in the light of biological research. *Rapp. P. V. Reun. Cons. Perm. Int. Explor. Mer* **20:** 1–228.

HOWELL, W. H. 1980. Temperature effects on growth and yolk utilization in yellowtail flounder, *Limanda ferruginea,* yolk-sac larvae. *Fish. Bull. (U.S.)* **78:** 731–740.

ITZHAKI, R. F., AND D. M. GILL. 1964. A microbiuret method for estimating proteins. *Anal. Biochem.* **9:** 401–410.

IWAI, T. 1969. Fine structure of gut epithelial cells of larval and juvenile carp during absorption of fat and protein. *Arch. Hist. Jpn.* **30:** 183–199.

JOHNS, D. M., AND W. H. HOWELL. 1980. Yolk utilization in summer flounder (*Paralichthys dentatus*) embryos and larvae reared at two temperatures. *Mar. Ecol. Prog. Ser.* **2:** 1–8.

KREFFT, G. 1961. A contribution to the reproductive biology of *Helicolenus dactylopterus* (De la Roche, 1809) with remarks on the evolution of the Sebastinae. *Rapp. P. V. Reun. Cons. Perm. Int. Explor. Mer* **150:** 243–244.

KUSAKARI, M. 1978. Pp. 117–139 in *Annual Report of the Hokkaido Institute of Mariculture.*

KUSAKARI, M. Y., O. MORI, AND K. KUDO. 1977. Studies on the breeding habit of a rock fish, *Sebastes schlegeli* (Hilgendorf). 2. On the breeding behavior of pregnant fish and the just spawned larvae. *J. Hokkaido Fish. Exp. Stn.* **34:** 1–11.

LAROCHE, W. A., AND S. L. RICHARDSON. 1980. Development and occurrence of larval and juveniles of the rockfishes *Sebastes flavidus* and *Sebastes melanops* (Scorpaenidae) off Oregon. *Fish. Bull. (U.S.)* **77:** 901–924.

LASKER, R. 1962. Efficiency and rate of yolk utilization by developing embryos and larvae of the Pacific sardine *Sardinops caerulea* (Girard). *J. Fish. Res. Board Can.* **19:** 867–875.

LAURENCE, G. C. 1973. Influence of temperature on energy utilization of embryonic and prolarval tautog, *Tautoga onitis*. *J. Fish. Res. Board Can.* **30:** 435–442.

LENARZ, W. H. 1973. Dependence of catch rates on size of fish larvae. *Rapp. P. V. Reun. Cons. Perm. Int. Explor. Mer* **164:** 270–275.

LOMBARDI, J., AND J. P. WOURMS. 1978. SEM of trophotaeniae, embryonic adaptations in an ophidioid, *Microbrotula randalli* and other viviparous fishes. *Am. Zool.* **18:** 607.

MACGREGOR, J. S. 1970. Fecundity, multiple spawning, and description of the gonads in *Sebastodes*. *U. S. Fish Wildl. Serv., Spec. Sci. Rep. Fish.* **596:** 12 pp.

MARR, J. C. 1956. The "critical period" in the early life history of marine fishes. *J. Cons. Perm. Int. Explor. Mer* **21:** 160–170.

MCCLURE, R. E. 1982. Neritic reef fishes off central Oregon: Aspects of life histories and the recreational fishery. M.S. Thesis, Oregon State University, Corvallis, Oregon.

MOSER, H. G. 1967a. Reproduction and development of *Sebastodes paucispinnis* and comparison with other rockfishes off Southern California. *Copeia* **1967:** 773–797.

MOSER, H. G. 1967b. Seasonal histological changes in the gonads of *Sebastodes paucispinnis* Ayres, an ovoviviparous teleost (Family Scorpanidae). *J. Morphol.* **123:** 329–354.

MOSER, H. G., AND E. H. AHLSTROM. 1978. Larvae and pelagic juveniles of blackgill rockfish, *Sebastes melanostomus*, taken in midwater trawls off southern California and Baja California. *J. Fish. Res. Board Can.* **35:** 981–996.

MOSER, H. G., E. H. AHLSTROM, AND E. M. SANDKNOP. 1977. Guide to the identification of scorpionfish larvae (Family Scorpaenidae) in the eastern Pacific with comparative notes on species of *Sebastes* and *Helicolenus* from other oceans. *NOAA Tech. Rep. NMFS CIRC*-**402,** 71 pp.

MOSER, H. G., AND J. L. BUTLER. 1981. Description of reared larvae and early juveniles of the calico rockfish, *Sebastes dalli* (Eigenmann and Beeson 1894). *CalCOFI Rep.* **2:** 88–95.

MOULTON, L. L. 1975. Life history observations of the Puget Sound rockfish *Sebastes emphaeus* (Starks 1911). *J. Fish. Res. Board Can.* **32:** 1439–1442.

NEEDHAM, J. 1931. *Chemical Embryology.* Cambridge, England. 2021 pp.

NEEDHAM, J. 1942. *Biochemistry and Morphogenesis.* Cambridge Univ. Press, London and New York. 787 pp.

NIIMI, A. J. 1972. Total nitrogen, nonprotein nitrogen, and protein content in largemouth bass (*Micropterus salmoides*) with reference to quantitative protein estimates. *Can. J. Zool.* **50:** 1607–1610.

O'CONNELL, C. P. 1976. Histological criteria for diagnosing the starving condition in early post yolk sac larvae of the northern anchovy, *Engraulis mordax* Girard. *J. Exp. Mar. Biol. Ecol.* **25:** 285–312.

OPPENHEIMER, J. M. 1937. The normal stages of *Fundulus heteroclitus*. *Anat. Rec.* **68:** 1–15.

PHILLIPS, J. B. 1964. Life history studies on ten species of rockfish (genus *Sebastodes*). *Calif. Dep. Fish Game Fish Bull.* **126:** 70 pp.

ROBERTS, R. J., M. BELL, AND H. YOUNG. 1973. Studies on the skin of plaice (*Pleuronectes platessa* L.) II. The development of larval plaice skin. *J. Fish Biol.* **5:** 103–108.

ROBERTSON, D. A. 1974. Developmental energetics of southern pigfish (Teleostei: Congriopodidae). *N. Z. J. Mar. Freshwater Res.* **8:** 611–620.

ROGERS, B. A., AND D. T. WESTIN. 1981. Laboratory studies on effects of temperature and delayed initial feeding on development of striped bass larvae. *Trans. Am. Fish. Soc.* **110:** 100–110.

SALONEN, K., J. SARVALA, I. HAKALA, AND M. L. VILJANEN. 1976. The relation of energy and organic carbon in aquatic invertebrates. *Limnol. Oceanogr.* **21:** 724–730.

SCRIMSHAW, N. S. 1945. Embryonic development in poeciliid fishes. *Biol. Bull.* **88:** 233–246.

SHIMIZU, M., AND J. YAMADA. 1980. Ultrastructural aspects of yolk absorption in the vitelline syncytium of the embryonic rockfish, *Sebastes schlegeli*. *Jpn. J. Ichthyol.* **27:** 56–63.

SMITH, C. L., C. S. RAND, B. SCHAEFFER, AND J. W. ATZ. 1975. *Latimeria*, the living coelacanth, is ovoviviparous. *Science* **190:** 1105–1106.

SMITH, P. E. 1973. The morality and dispersal of sardine eggs and larvae. *Rapp. R. P. Reun. Cons. Perm. Int. Explor. Mer* **164:** 281–292.

SOROKIN, V. P. 1961. The redfish; gametogenesis and migrations of the *Sebastes marinus* (L.) and *Sebastes mentella* Travin. *Rapp. P. V. Reun. Cons. Perm. Int. Explor. Mer* **150:** 245–250.

TANING, A. V. 1961. Larval and postlarval stages of *Sebastes* species and *Helicolenus dactylopterus*. *Rapp. P. V. Reun. Cons. Perm. Int. Explor. Mer* **150:** 234–240.

THIBAULT, R. E., AND R. J. SCHULTZ. 1978. Reproduction adaptations among viviparous fishes (Cyprinidontiformes: Poeciliidae). *Evolution* **32:** 320–333.

TSUKAHARA, H. 1962. Studies on habits of coastal fishes in the Amakusa Islands. 2. Early life history of the rockfish *Sebastiscus marmoratus* (Cuvier et Valenciennes). *Rec. Oceanogr. Works. Jpn. Spec.* **6:** 49–55.

TURNER, C. L. 1937. The trophotaenidae of the Goodeidae, a family of viviparous cyprinodont fishes. *J. Morphol.* **61:** 495–523.

TURNER, C. L. 1947. Viviparity in teleost fishes. *Sci. Mon.* **65:** 508–518.

UMBREIT, W. W., R. H. BURRIS, AND J. F. STAUFFER. 1972. *Manometric and Biochemical Techniques.* Burgess Publ. Co., Minneapolis. 387 pp.

VEITH, W. J. 1979. The chemical composition of the follicular fluid of the viviparous teleost *Clinus superciliosus*. *Comp. Biochem. Physiol.* **63A:** 37–40.

VEITH, W. J. 1980. Viviparity and embryonic adaptations in the teleost *Clinus superciliosus*. *Can. J. Zool.* **58:** 1–12.

WARE, D. M. 1975. Relation between egg size, growth, and natural mortality of larval fish. *J. Fish. Res. Board Can.* **32:** 2503–2512.

WATANABE, Y. 1981. Ingestion of horseradish peroxidase by the intestinal cells in larvae of juveniles of some teleosts. *Bull. Jpn. Soc. Sci. Fish.* **47:** 1299–1307.

WATANABE, Y. 1982. Intracellular digestion of horseradish peroxidase by the intestinal cells of teleost larvae and juveniles. *Bull. Jpn. Soc. Sci. Fish.* **48:** 37–42.

WEBB, P. W., AND J. R. BRETT. 1972. Respiratory adaptations of prenatal young in the ovary of two species of viviparous seaperch, *Rhacochilus vacca* and *Embiotoca lateralis*. *J. Fish. Res. Board Can.* **29:** 1525–1542.

WEIHS, D. 1980. Respiration and depth control as possible reasons for swimming of northern anchovy, *Engraulis mordax*, larvae. *Fish. Bull. (U. S.)* **78:** 109–118.

WOURMS, J. P. 1981. Viviparity: The maternal-fetal relationship in fishes. *Am. Zool.* **21:** 473–515.

WOURMS, J. P., AND O. BAYNE. 1973. Development of the viviparous brotulid fish, *Dinematichthys illucoeteoides*. *Copeia* **1973:** 32–40.

WOURMS, J. P., AND D. COHEN. 1975. Trophotaeniae, embryonic adaptations in the viviparous ophidioid fish, *Oligopus longhursti:* A study of museum specimens. *J. Morphol.* **147:** 385–401.

WOURMS, J. P., M. D. STRIBLING, AND J. W. ATZ. 1980. Maternal fetal nutrient relationships in the coelacanth, *Latimeria*. *Am. Zool.* **20:** 962.

YAMADA, J. 1968. A study on the structure of surface cell layers in the epidermis of some teleosts. *Annot. Zool. Jpn.* **41:** 1–8.

Reference: *Biol. Bull.* **167:** 371–377. (October, 1984)

PROTEIN SYNTHESIS IN NORMAL AND LOBELESS GASTRULAE OF *ILYANASSA OBSOLETA*

J. R. COLLIER

Biology Department, Brooklyn College, Brooklyn, New York 11210 and The Graduate School, The City University of New York

ABSTRACT

Several hundred proteins synthesized by normal and lobeless *Ilyanassa* gastrulae were identified by the two-dimensional electrophoresis of polypeptides labeled *in vivo* with ^{35}S-methionine. Acidic proteins were separated in the first dimension by isoelectric focusing and basic proteins by non-equilibrium pH gradient electrophoresis (NEPHGE).

No qualitative differences were detected among either the acidic or basic polypeptides produced by normal or lobeless gastrulae. These findings show, for those peptides detected in this analysis, that (1) the stage-specific changes in protein synthesis that occur in *Ilyanassa* embryos by gastrulation (Collier and McCarthy, 1981) are not polar lobe dependent, and (2) the polar lobe cytoplasm does not qualitatively affect the expression of either maternal or embryonic mRNAs during gastrulation.

INTRODUCTION

Previous observations on protein synthesis during the embryogenesis of the marine mud snail *Ilyanassa obsoleta* have been restricted to early and late cleavage stages (Brandhorst and Newrock, 1981; Collier, 1981a; Collier and McCarthy, 1981) or to later stages of organogenesis (Collier, 1983). Major qualitative and quantitative changes in protein synthesis occur during *Ilyanassa* embryogenesis between the 4- and 25-cell stages of development (Collier and McCarthy, 1981). However, it is not clear whether removal of the polar lobe, a vegetal region of egg cytoplasm essential for normal development, influences gastrular protein synthesis. An answer to this question is important because maternal mRNAs that may have been segregated into different regions of the embryo may not be expressed until a later stage of development, such as gastrulation.

This study clarifies the role of the polar lobe in gastrular protein synthesis. Observations on the basic proteins synthesized by normal and lobeless gastrulae are also included.

MATERIALS AND METHODS

Maintenance of snails and rearing of embryos

Snails collected from Plumb Beach in Brooklyn, New York were maintained at Brooklyn College in tanks of recirculating sea water. When fed a fresh clam on alternate days the snails laid fresh egg capsules daily. Embryos were reared at 19°C in filtered sea water (obtained from the Supply Department of the Marine Biological Laboratory, Woods Hole, Massachusetts) containing 200 micrograms per ml of streptomycin and penicillin. Polar lobes were isolated from eggs in the trefoil stage by

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agitation in Ca-Mg-low sea water (Collier, 1981b). Normal and lobeless embryos were obtained by pooling eggs from several egg capsules.

Labeling of proteins and sample preparation

Proteins were labeled *in vivo* by incubating 200 to 400 embryos at 19°C for 4 h in 120 microcuries per ml of L ^{35}S-methionine (New England Nuclear, 800–1100 Ci/mmole). After incubation with radioactive precursor the embryos were rinsed in Ca-Mg-free sea water, homogenized with a Dounce homogenizer in 0.02 *M* Tris, pH 8.8, containing 0.002 *M* $CaCl_2$ and 0.05 mg/ml micrococcal nuclease, incubated at room temperature for 3 minutes, made 2% with mercaptoethanol, quick frozen, and lyophilized. The lyophilized homogenate was dissolved in 0.1 ml of sample buffer containing 8.5 *M* urea (Schwarz-Mann Ultrapure), 2.0% NP-40 (Tergito, type NP-40 from Sigma Chemical Co.), 0.1 *M* dithiothreitol, 0.4% pH 3–10 ampholyte, 1.6% pH 5–7 ampholyte (all ampholytes were from Bio-Rad Laboratories), and stored at −70°C.

The radioactivity of all samples was determined following precipitation with trichloroacetic acid (TCA). The acid precipitates of protein were washed with TCA, collected on nitrocellulose membranes (Millipore Co.), and counted in a scintillation spectrometer.

Electrophoresis

Samples of protein frozen in sample buffer were thawed, and electrophoresis in the first dimension (isoelectric focusing) was carried out as described by O'Farrell (1975) with a final ampholyte concentration of 2.8% (1.6% pH 5–7; 0.4% pH 3–10; 0.4% pH 3–5; and 0.4% pH 7–9). Focusing gels were polymerized and run in 20 × 0.15 cm glass tubes and electrophoresis was at 25°C for 19,000 volt hours. These focusing gels had a pH gradient from 5.0 to 7.0.

Basic proteins were separated in the first dimension by nonequilibrium pH gradient electrophoresis (NEPHGE) as described by O'Farrell *et al.* (1977). For NEPHGE electrophoresis sample preparation, composition, and size of focusig gel was as described above except for changes in the arrangement of buffer reservoirs and the polarity of electrophoresis. The cathode buffer chamber containing 0.02 *N* NaOH was placed on the bottom of the electrophoresis unit, and the anode buffer chamber containing 0.01 *N* phosphoric acid was placed at the top of the electrophoresis unit. The sample was loaded at the anode end of the gel and during electrophoresis the basic proteins migrated into the gel and toward the cathode, rather than into the cathode buffer chamber as in isoelectric focusing. Further, electrophoresis was continued for only 1200 VH, *i.e.*, much less than required to reach equilibrium, in order to retain the basic proteins on the gel. The gels used for isoelectric focusing had an effective pH gradient from 7.3 to 8.7.

Second dimension electrophoresis was on 10% polyacrylamide separating gels prepared and run according to Laemmli (1970). Gels were prepared for fluorography as described by Garrells (1979), dried, and exposed to Kodak X-Omat film for the times indicated in the figure legends.

Results

In *Ilyanassa* the gastrula is formed by epiboly of the micromeres over the yolky macromeres. Gastrulation, as determined by the maximal closure of the blastopore, is completed after about 54 hours of development at 19°C (Collier, 1976). When the

polar lobe is removed from *Ilyanassa* eggs the time and mode of gastrulation is essentially the same as in the normal embryo.

Segregation of ectoderm, mesoderm, and endoderm occurs shortly after the formation of the primary mesentoblast cell (the 4d cell), which appears after one day of development at 19°C. The morphogenetic movements that form the gastrula are complete just after the second day of development as described above. Collier and McCarthy, (1981) suggested that the 24-hour embryo, which has approximately 29 cells, be called a mesentoblast embryo and that the older embryo which has completed the morphogenetic movements required for the formation of the blastopore be called a gastrula.

Figures 1A, B are autofluorographs of radioactive polypeptides produced, respectively, by normal and lobeless *Ilyanassa* gastrulae. These radioactive peptides were separated by two-dimensional electrophoresis on polyacrylamide gels. They were extracted separately from control and lobeless embryos that were reared to gastrulae and incubated in ^{35}S-methionine. Some apparent discrepancies among the radioactive spots in these fluorographs are indicated by arrows in Figure 1A. For example, spots that appear to be present in Figure 1A but absent in Figure 1B are indicated by long arrows, and one spot smeared in Figure 1B but clearly resolved in Figure 1A is marked by a short arrow. These discrepancies are artifacts because they were not observed consistently among gels prepared from different protein preparations nor among replicate electrophoretic separations from the same protein sample.

About 685 individual radioactive spots are resolved on each of the autofluorographs in Figures 1A, B. These spots correspond to polypeptides that were translated from the more abundant classes of mRNAs of normal and lobeless *Ilyanassa* gastrulae. That this is only a partial detection of translatable mRNAs is evident from considerations of the sensitivity of resolution and detection of radioactive peptides by two-dimensional electrophoresis, the distribution of methionine among proteins, and the limits of the pH gradient, *i.e.*, pH 5 to 7, of the isoelectric focusing gels. For example, in regard to the latter point it has been estimated by Tufaro and Brandhorst (1979) that more than 85% of ^{35}S-methionine-labeled sea urchin proteins enter isoelectric focusing gels in the pH range of 4.5 to 7.2. Thus, the observations (Figs. 1A, B) on proteins separated from several groups of normal and lobeless *Ilyanassa* gastrulae demonstrate the similarity of proteins produced by these two classes of embryos at gastrulation.

Figures 2A, B are autofluorographs of *in vivo* labeled proteins extracted from normal and lobeless gastrulae, respectively, and resolved by NEPHGE. This method of electrophoresis resolves basic proteins that either do not enter or fail to be resolved in isoelectric focusing gels with acidic and neutral pH gradients. Because NEPHGE gels are not focused to equilibrium, only proteins in the basic regions (pH 7.3–8.7) of the gel are resolved. Although this pH gradient extends more into the alkaline pH range than the gradient used in equilibrium isoelectric focusing, it is not satisfactory for the separation of histones, which would, in the presence of urea, require a gradient from pH 8.9 to 10.25 (Valkonen and Piha, 1980). Gels focused to equilibrium show some apparent differences among the radioactive spots displayed in Figure 2. However, these discrepancies were not seen consistently among replicate gels. Therefore, this analysis shows the similarities of the basic polypeptides produced by normal and lobeless embryos.

Discussion

If maternal mRNAs are differentially expressed, either by differential localization, translation, or destruction, in different blastomeres or regions of the embryo (Tufaro

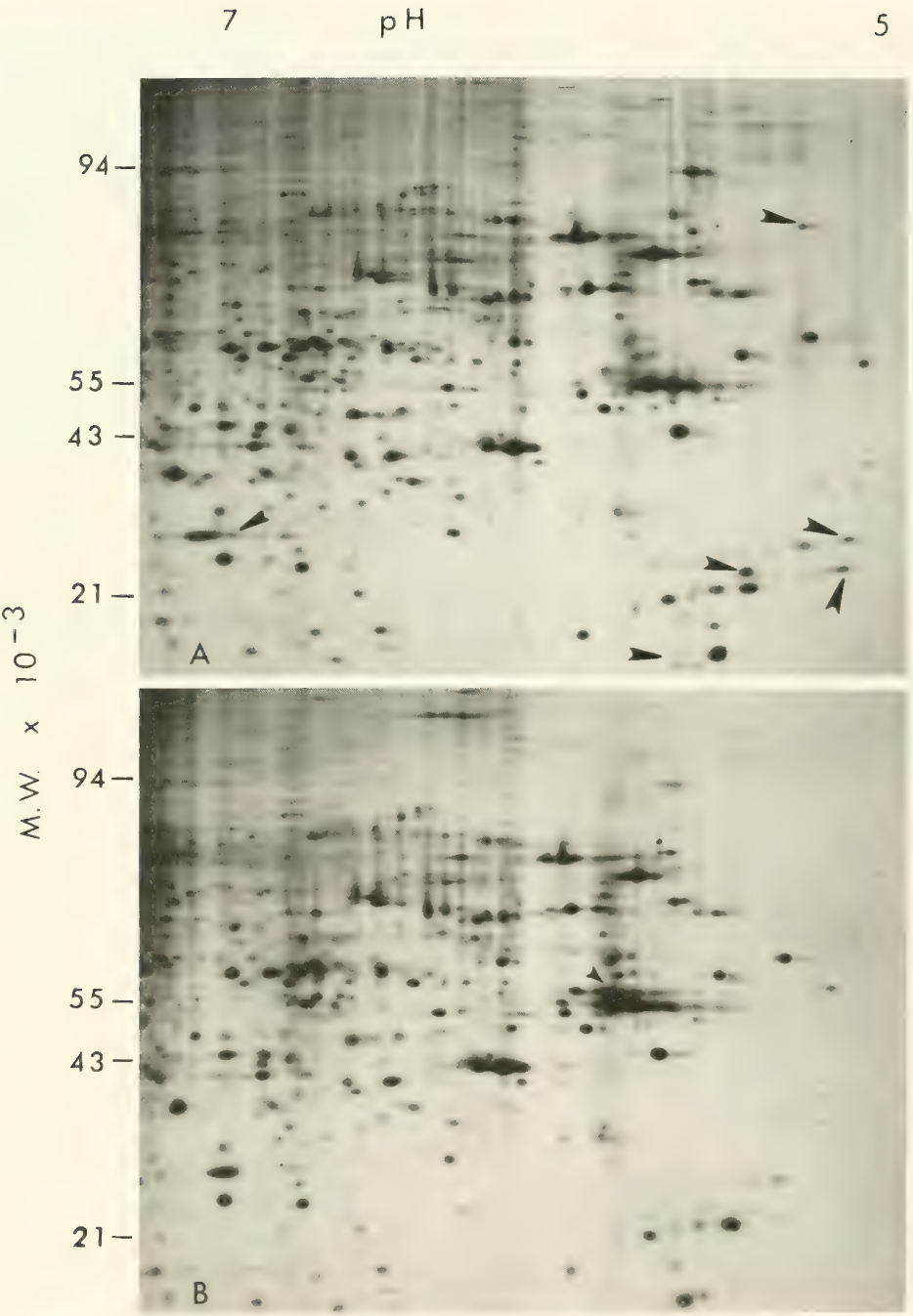

FIGURE 1. Autoradiographs of ^{35}S-methionine labeled acidic proteins synthesized by (A) normal, and (B) lobeless gastrulae. The large arrows in 1A indicate spots that were not seen consistently among replicate gels. The small arrow in 1B marks a spot smeared in this figure but clearly resolved in 1A.

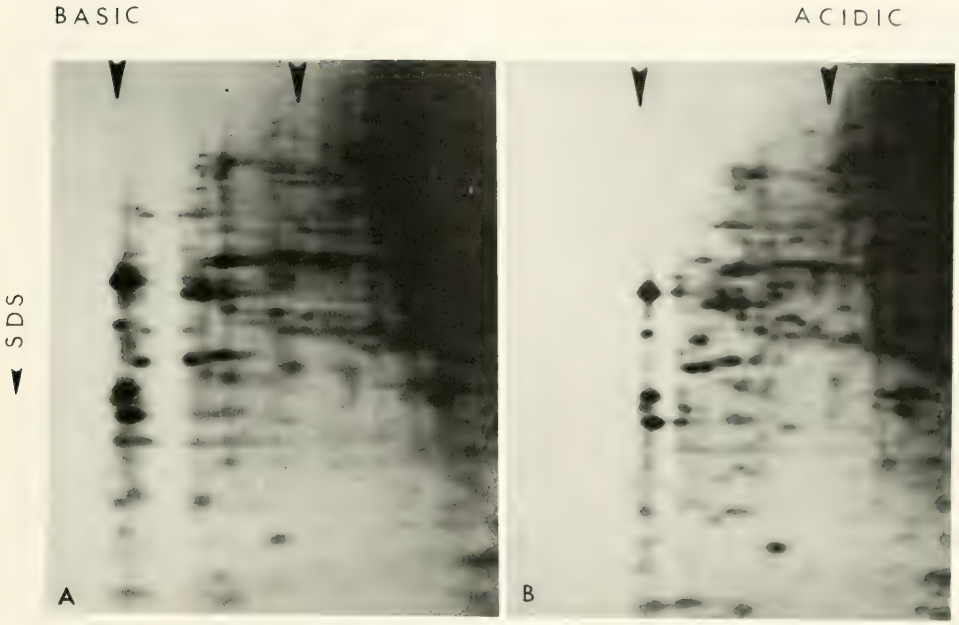

FIGURE 2. Autoradiographs of ^{35}S-methionine labeled basic proteins synthesized by (A) normal, and (B) lobeless gastrulae. Vertical arrows mark the basic region (pH 7.3–8.7) of the NEPHGE autoradiograph in which polypeptides were resolved.

and Brandhorst, 1979; Brandhorst *et al.*, 1983; Collier, 1983; Jeffery, 1983), one may expect that this expression could occur at any of several stages of development. Analyses of protein synthesis during early stages of *Ilyanassa* development have failed to detect a differential expression of mRNAs. Therefore it became important to ask if the expression of some mRNAs may have been delayed until a later period in development, *e.g.*, gastrulation.

The results reported here demonstrated no detectable qualitative differences among 685 polypeptides produced by normal and lobeless gastrulae. A number of basic non-histone proteins were also found to be similarly produced by both normal and lobeless gastrulae. All of these proteins were translated from the prevalent and moderately prevalent classes of gastrular mRNAs. They do not include proteins coded for by the rare or complex classes of mRNA which are not detected by two-dimensional electrophoresis of proteins labeled *in vivo* with ^{35}S-methionine. Therefore, it is clear that the polar lobe cytoplasm does not have a delayed effect on the expression of maternal mRNAs. Similarly, the polar lobe does not qualitatively influence the expression of embryonic mRNAs that are produced and translated during gastrulation.

From these observations it may also be deduced that the stage-specific changes in protein synthesis that occur in the *Ilyanassa* embryo prior to gastrulation (Collier and McCarthy, 1981) are not dependent on the polar lobe cytoplasm because these changes occur in both normal and lobeless gastrulae.

These and earlier studies of protein synthesis in *Ilyanassa* embryos, have shown no obvious correlation between qualitative changes in protein synthesis and the developmental events influenced by the egg cytoplasm in the polar lobe. Earlier studies (Donohoo and Kafatos, 1973; Newrock and Raff, 1975) on protein synthesis in *Ilyanassa*, which suggested a differential expression of maternal mRNAs, have not

been confirmed by the recent work of Brandhorst and Newrock (1981) and Collier and McCarthy (1981). The observations of Cheney and Ruderman (1978) on *Spisula* embryos have not been published in sufficient detail for appraisal. No qualitative differences in the pattern of protein synthesis have been detected during determinative stages of sea urchin embryogenesis, *e.g.*, the micro-, meso-, and macromeres of the 16-cell (Tufaro and Brandhorst, 1979) and vegetalized sea urchin embryos (Hutchins and Brandhorst, 1979). Therefore, the available data suggest that the developmental events that implement determination are not reflected by qualitative changes in the synthesis of the abundant classes of proteins which are readily detectable by two-dimensional electrophoresis of *in vivo* labeled proteins. However, the finding of posterior indicator and head foretelling proteins in *Smittia* embryos by Jackle and Kalthoff (1980, 1981) are in striking contrast to the absence of qualitative changes in protein synthesis during the determinative stages of *Ilyanassa* and sea urchin embryos.

In a previous discussion (Collier, 1983) of the relation of quantitative differences in protein synthesis to differentiation, it was pointed out that the greatest quantitative disparity in protein synthesis between normal and lobeless embryos was among the most abundant proteins, *i.e.*, those most rapidly labeled. This observation, as well as those of Harkey and Whiteley (1982) on sea urchin embryos, shows that the major quantitative differences that occur during differentiation and organogenesis are developmentally regulated. In view of the failure to establish a correlation between qualitative changes of this class of proteins and differentiation, it is reasonable to think that these proteins are ubiquitously required by all cells and that their role in differentiation is achieved by quantitative variations among different cell types.

If the determinative events of early embryogenesis involve the differential localization of maternal or embryonic mRNAs and the prevalent classes of mRNA are excluded, then one must search for these determinants among the rare and complex class of mRNAs. The rarity and complexity of these mRNAs, as estimated for sea urchin embryos (Galau *et al.*, 1976; Davidson and Britten, 1979; and Duncan and Humphreys, 1981), are, respectively, 1–15 copies per cell and 12,000 to 14,000 individual species of mRNAs. To approach this problem requires subfractionation of proteins prior to electrophoresis in order to resolve 12,000 to 14,000 radioactive spots on 150 to 200 square centimeters of polyacrylamide and the removal of the abundant class of proteins, which obscures the registry of the rare species of labeled proteins on an autoradiograph. Finally, the distribution of oogenetic proteins among the early blastomeres should be considered in a search for molecules related to determination.

Acknowledgments

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LITERATURE CITED

Bedard, P.-A., and B. P. Brandhorst. 1983. Patterns of protein synthesis and metabolism during sea urchin embryogenesis. *Dev. Biol.* **96:** 74–83.

Brandhorst, B. P., and K. M. Newrock. 1981. Post-transcriptional regulation of protein synthesis in *Ilyanassa* embryos and isolated polar lobes. *Dev. Biol.* **83:** 250–254.

Cheney, C. M., and J. V. Ruderman. 1978. Segregation of maternal mRNA at first cleavage in embryos of the mollusc *Spisula solidissima*. *J. Cell Biol.* **79:** 349a.

Collier, J. R. 1976. Nucleic acid chemistry of the *Ilyanassa* embryo. *Am. Zool.* **16:** 483–500.

Collier, J. R. 1981a. Protein synthesis in the polar lobe and lobeless egg of *Ilyanassa obsoleta*. *Biol. Bull.* **160:** 366–375.

COLLIER, J. R. 1981b. Methods of obtaining and handling eggs and embryos of the marine mud snail *Ilyanassa obsoleta*. Pp. 217–232 in *Marine Invertebrates*. Report of the Committee on Marine Invertebrates, Institute of Laboratory Animal Resources, National Research Council, National Academy Press, Washington, DC.

COLLIER, J. R. 1983a. Protein synthesis during *Ilyanassa* organogenesis. *Dev. Biol.* **100**: 256–259.

COLLIER, J. R. 1983b. The biochemistry of molluscan development. Pp. 253–297 in *The Mollusca*, Vol. 3, *Development*, N. H. Verdonk, J. A. M. Van Den Biggelaar, and A. S. Topma, eds. Academic Press, NY.

COLLIER, J. R., AND M. E. MCCARTHY. 1981. Regulation of polypeptide synthesis during early embryogenesis of *Ilyanassa obsoleta*. *Differentiation* **19**: 31–46.

DAVIDSON, E. H., AND R. J. BRITTEN. 1979. Regulation of gene expression: possible role of repetitive sequences. *Science* **204**: 1052–1059.

DONOHOO, P., AND F. C. KAFATOS. 1973. Differences in the proteins synthesized by the progeny of the first two blastomeres of *Ilyanassa*, a "mosiac" embryo. *Dev. Biol.* **32**: 224–229.

DUNCAN, R., AND T. HUMPHREYS. 1981. Most sea urchin maternal mRNA sequences in every abundance class appear in both polyadenylated and nonpolyadenylated molecules. *Dev. Biol.* **88**: 201–210.

GALAU, G. A., W. H. KLEIN, M. M. DAVIS, B. J. WOLD, R. J. BRITTEN, AND E. H. DAVIDSON. 1976. Structural gene sets active in embryos and adult tissues of the sea urchin. *Cell* **7**: 487–505.

GARRELS, J. I. 1979. Two-dimensional gel electrophoresis and computer analysis of proteins synthesized by clonal cell lines. *J. Biol. Chem.* **254**: 7961–7977.

HARKEY, M. A., AND A. H. WHITELEY. 1982. Cell-specific regulation of protein synthesis in the sea urchin gastrula: A two-dimensional electrophoretic study. *Dev. Biol.* **93**: 453–462.

HUTCHINS, R., AND B. P. BRANDHORST. 1979. Commitment to vegetalized development in sea urchin embryos. *Wilhelm Roux's Arch. Dev. Biol.* **186**: 95–102.

JACKLE, H., AND K. KALTHOFF. 1980. Synthesis of a posterior indicator protein in normal embryos and double abdomens of *Smittia* sp. (Chironomidae, Diptera). *Proc. Natl. Acad. Sci.* **77**: 6700–6704.

JACKLE, H., AND K. KALTHOFF. 1981. Proteins fortelling head or abdomen development in the embryo of *Smittia* spec. (Chironomidae, Diptera). *Dev. Biol.* **85**: 287–298.

JEFFERY, W. R. 1983. Maternal mRNA and the localization problem. *Control of Embryonic Gene Expression*. M. A. Q. Siddiqui, ed. CRC Press, Boca Raton, FL.

LAEMMLI, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680–685.

NEWROCK, K. M., AND R. A. RAFF. 1975. Polar lobe specific regulation of translation in embryos of *Ilyanassa obsoleta*. *Dev. Biol.* **42**: 242–261.

O'FARRELL, P. H. 1975. High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* **250**: 4007–4021.

O'FARRELL, P. Z., H. M. GOODMAN, AND P. H. O'FARRELL. 1977. High resolution two-dimensional electrophoresis of basic as well as acidic proteins. *Cell* **12**: 1133–1142.

TUFARO, F., AND B. P. BRANDHORST. 1979. Similarity of proteins synthesized by isolated blastomeres of early sea urchin embryos. *Dev. Biol.* **72**: 390–397.

VALKONEN, K. H., AND R. S. PIHA. 1980. Isoelectric focusing and isoelectric points of bovine liver histones. *Anal. Biochem.* **104**: 499–505.

Reference: *Biol. Bull.* **167**: 378–389. (October, 1984)

FORMATION, ORGANIZATION, AND COMPOSITION OF THE EGG CAPSULE OF THE MARINE GASTROPOD, *ILYANASSA OBSOLETA*

CHARLES H. SULLIVAN[1] AND TIMOTHY K. MAUGEL

Laboratory for Ultrastructural Research, Department of Zoology, University of Maryland, College Park, Maryland 20742

ABSTRACT

Embryos of the marine mud snail *Ilyanassa obsoleta* undergo early development within an egg capsule. After about a week of encapsulation, embryos hatch by releasing a chemical substance that removes the plug found at the apex of a capsule. However, the mechanism of action of this hatching substance remains poorly understood. To study how the hatching substance functions, we examined the composition of the egg capsule, particularly the plug region, to determine what the "substrate" of the hatching substance might be. We have also examined the formation and organization of the egg capsule to determine the origin and identity of the regions of a capsule that the hatching substance must remove. The results show that the *Ilyanassa* egg capsule is organized into four layers, the outer three of which are composed of protein and carbohydrate. Portions of the two inner layers of the capsule wall extend into the capsule apex and form the plug, which is dissolved by the hatching substance. The isolated capsule plug region contains three major glycoproteins resolved on sodium dodecyl sulfate-polyacrylamide gels. Therefore, the hatching substance may be a protease similar in action to the enzymes released by many other embryos at hatching.

INTRODUCTION

The egg capsules of prosobranch gastropod molluscs are examples of the various types of extracellular envelopes that surround the embryos of animals. Egg capsules are produced in the maternal oviduct by a process described in detail for four species of gastropods by Fretter (1941). The process may be similar for other species as well. Eggs are released from the ovary and are fertilized in the upper regions of the oviduct. The fertilized eggs, in groups of up to several hundred, move down the oviduct to a specialized region, the albumen gland, and are embedded in the secretions of this gland. A sphincter between the albumen gland and the next region of the oviduct, the capsule gland, relaxes and the embryos are forced into the protein and mucus secretions of the capsule gland. This movement of the embryos leaves an opening in the secretions that is subsequently sealed by the operculum. Ultimately, the soft capsule passes out of the oviduct, through the mantle cavity, and along a temporary groove on the right side of the foot to the ventral pedal gland. Within this latter gland, the capsule is sculptured into a species-specific shape, hardened, and attached to a substrate.

The time an embryo spends within the egg capsule is variable (Fretter and Graham, 1962). Some gastropod embryos emerge within a week as swimming premetamorphic veliger larvae (Scheltema, 1967), so that hatching can be a relatively early developmental

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[1] Present Address: Department of Biology, Gilmer Hall, University of Virginia, Charlottesville, Virginia 22901.

Send all correspondence to Dr. Sullivan at the present address.

event. Other snails crawl out of capsules as postmetamorphic juveniles, after three to four months of encapsulation (Harasewych, 1978).

Three different hatching mechanisms have been described for gastropods. First, some egg capsules take up water and rupture as a result of an increased internal osmotic pressure. Capsules of *Littorina littorea* swell from an average diameter of 156 μm to 229 μm just prior to hatching, and hatching can be accelerated in dilute sea water or slowed in concentrated sea water (Davis, 1968). Second, some embryos use parts of their bodies to mechanically tear the capsule. *Lymnaea stagnalis* embryos begin normal feeding movements of the radula to rasp a hole in the capsule wall (Vaughn, 1953). Third, some gastropods escape from the capsule by chemically dissolving a pre-designated region of the capsule, termed the operculum or plug. Chemical hatching has been described for several gastropod embryos, including the oyster drill, *Urosalpinx cinerea* (Hancock, 1956), the whelks *Busycon carica* and *B. canaliculatum* (Harasewych, 1978), and the mud snail, *Ilyanassa obsoleta* (Pechenik, 1975).

Chemical hatching from other types of extraembryonic envelopes (fertilization envelope, chorion) as in sea urchins, amphibians, and fish, has been studied in detail. In these systems, the hatching enzymes are proteases that digest proteins of the envelope surrounding an embryo (see Sullivan and Bonar, 1984). However, the nature of the hatching substances released by gastropod embryos and how they function in the hatching process remain largely unknown.

In the present study, the organization of the egg capsule of *Ilyanassa obsoleta* was examined to determine how the capsule is formed and to identify the components of the capsule that the hatching substance dissolves. The *Ilyanassa* capsule is composed of four structural layers (L1–L4) that are secreted by cells of the oviduct. The capsule is covered by an outer fibrous layer (L0) deposited by cells of the ventral pedal gland. Several of these layers are located in the capsule apex and pose a barrier to hatching. The three outer layers of the capsule wall (L1, L2, and L3) and the capsule plug, a continuation of L3, are composed of protein and carbohydrate. Isolated capsule plugs contain three major glycoproteins having molecular weights of 49,000, 29,000, and 24,000 daltons. Because the hatching substance dissolves protein and carbohydrate components of the capsule plug region, it may be a protease or carbohydrase.

Materials and Methods

Biological material

Ilyanassa adults were collected from mud flats near Lewes, Delaware or in Woods Hole, Massachusetts. The snails were maintained in artificial sea water (Instant Ocean) at 10°C. To initiate reproduction, 35–40 adults were transferred to a five gallon aquarium containing aerated Instant Ocean (pH 8.0, 28‰) at 23°C, and were fed fresh clam meat regularly. Within a few days, egg capsules were produced. It was possible to observe the final stages of capsule formation (when the capsule passes from the mantle cavity to the foot) and collect capsules during this time. For the other studies, fully formed capsules were collected from the sides of the aquarium where females deposited capsules in great numbers. All capsules examined in this study were less than 24 hours old.

Electron microscopy

To prepare specimens for electron microscopy, newly collected capsules were cut open at the base and were washed repeatedly to remove embryos and capsule fluid. Capsule fragments were fixed for a minimum of 24 hours in 2.5% glutaraldehyde,

prepared in Millipore filtered sea water. Following several rinses in filtered sea water, samples prepared for transmission electron microscopy were postfixed in 1% aqueous OsO_4, dehydrated through an ethanol series, and embedded in Epon (Luft, 1961). Thin sections were cut with glass or diamond knives on a Sorvall MT-2B ultramicrotome, collected on 75 × 300 mesh copper grids (Pelco, Inc.), doubly stained with 2% aqueous uranyl acetate and 0.2% aqueous lead citrate (Venable and Coggeshall, 1965), and examined at 75 kV on a Hitachi HU-12 or at 50 kV on an RCA EMU-3H transmission electron microscope.

Capsules prepared for scanning electron microscopy were similarly fixed and washed repeatedly with distilled water, dehydrated through ethanol, and critical point dried from CO_2 (Denton DCP-1). Capsules were mounted on aluminum stubs, coated with gold-palladium (60:40), and viewed at 10 or 20 kV on an AMR 1000A scanning electron microscope. To view internal capsular structure, capsules were cut with razor blades prior to fixation or dehydrated capsules were frozen in liquid nitrogen and cryofractured with a cold razor blade before critical point drying (Humphreys *et al.*, 1974).

Histochemistry

Two approaches to determining the histochemical composition of the capsule were utilized. Capsules were fixed in 2.5% glutaraldehyde and prepared as described above for transmission electron microscopy except the postfixation in osmium was omitted. Thick (1 μm) sections of plastic embedded capsules were mounted on slides, the Epon removed in sodium methoxide (Steffens, 1978) and the sections stained, as described below. Alternatively, fresh capsules were fixed in Carnoy's fixative (Humason, 1979) and stained, then embedded in Epon and sectioned. Histochemical tests for protein (dinitrofluorobenzene), basic amino acids (fast green), aromatic amino acids (Baker's test), carbohydrates (periodic acid-Schiff's), acid mucopolysaccharides (alcian blue), metachromasia (toluidine blue), and lipid (sudan black) were performed (Pearse, 1968; Chayen *et al.*, 1973). Positive results with alcian blue that could be due to sulfate groups or carboxyl groups, were distinguished by including an increasing concentration of $MgCl_2$ in different preparations of 0.1% alcian blue (pH 2.5). Staining of sulfate groups occurs above 0.8 *M* $MgCl_2$ while staining of carboxyl groups occurs below 0.2 *M* $MgCl_2$. Both groups stain at intermediate concentrations of $MgCl_2$ (Pearse, 1968).

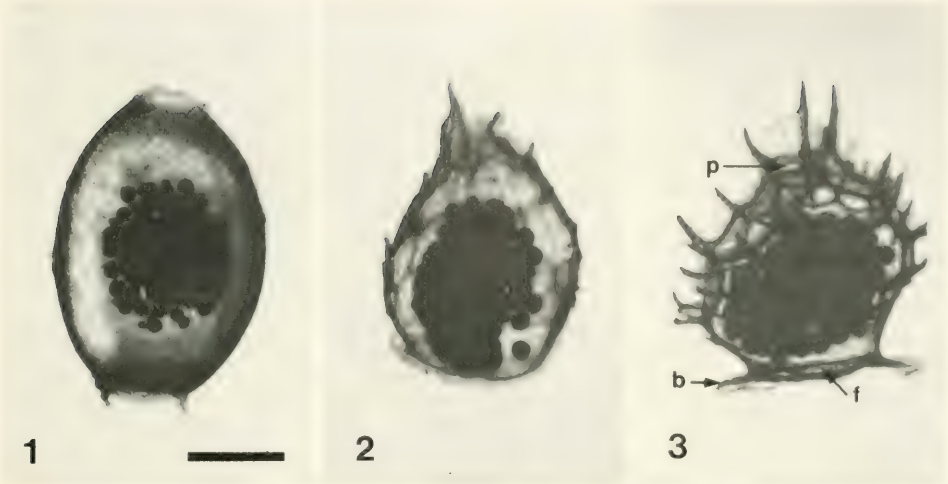
Polyacrylamide gel electrophoresis

Capsule plugs were dissected from approximately 60 newly deposited capsules, washed repeatedly in distilled water, and homogenized in 50 μl of 1% sodium dodecyl sulfate (SDS). The homogenate was added to an equal volume of 2× sample buffer (10% mercaptoethanol, 4% SDS, 20% glycerol, .002% bromophenol blue in 125 m*M* Tris, pH 6.8) and examined by SDS-polyacrylamide gel electrophoresis (Laemmli, 1970). Gels were stained for protein with Coomassie blue (Weber and Osborn, 1969) and for carbohydrate by the periodic acid-Schiff's stain (Fairbanks *et al.*, 1971). Molecular weight standards were co-electrophoresed with samples for determination of the molecular weights of plug proteins (Weber and Osborn, 1969).

RESULTS

Capsule organization

The final stages of capsule formation in *Ilyanassa* are very similar to those described for the closely related snail *Nassarius reticulatus* (Fretter, 1941). A biconvex, smooth



FIGURES 1–3. Light micrographs of the final steps of capsule formation in *Ilyanassa obsoleta*. A biconvex capsule (Fig. 1) emerges from the oviduct enclosing a mass of embryos and is passed to the pedal gland in the foot. A capsule remains in the pedal gland for 15–16 minutes, but after 7–8 minutes sculpturing of the capsule is underway (Fig. 2). The plug region (p), basal disk (b), and floor of the embryo chamber (f) are easily distinguished in the mature capsule (Fig. 3). Bar = 0.5 mm.

walled capsule emerges from the mantle cavity (Fig. 1), that is transferred to the pedal gland by muscular activity of the foot. Within the pedal gland, sculpturing of the capsule surface is already underway after eight minutes (Fig. 2) and by 15–16 minutes, a hardened and fully sculptured capsule emerges from the pedal gland (Fig. 3). Mature egg capsules vary from 1–3 mm in length and 1–2 mm in width. Although neither end of a capsule can be identified as "plug end" or "base" before a capsule enters the pedal gland, the end of the capsule deepest in the pedal gland becomes the apical end containing the plug, while the basal disk protrudes from the foot and is cemented to the substratum (Fig. 3).

The egg capsule wall consists of four distinct layers, designated L1–L4 (following the scheme described for *Urosalpinx* capsules by Tamarin and Carriker, 1967; Table I). These are visible when capsules are examined by transmission electron microscopy (Fig. 4). A "typical" egg capsule is 2 mm long and 1 mm wide, has a

TABLE I

Histochemical composition of the Ilyanassa *egg capsule*

Stain	Specificity	Result
Dinitrofluorobenzene	Proteins	All layers positive
Fast Green	Basic Amino Acids	L1, L2 positive
Baker's Test	Tyrosine	L1, L3, plug positive
Periodic Acid-Schiff's	Carbohydrates	All layers positive
Alcian Blue	Acid Mucopolysaccharides	
0–0.2 *M* $MgCl_2$	COOH groups	L3 and plug positive
0.2–0.6 *M* $MgCl_2$	SO_4 and COOH groups	L3 and plug positive
0.8 *M* $MgCl_2$	SO_4 groups	All layers negative
Toluidine Blue	Metachromasia of acidic groups	L1, L3, plug positive
Sudan Black	Lipids	All layers negative

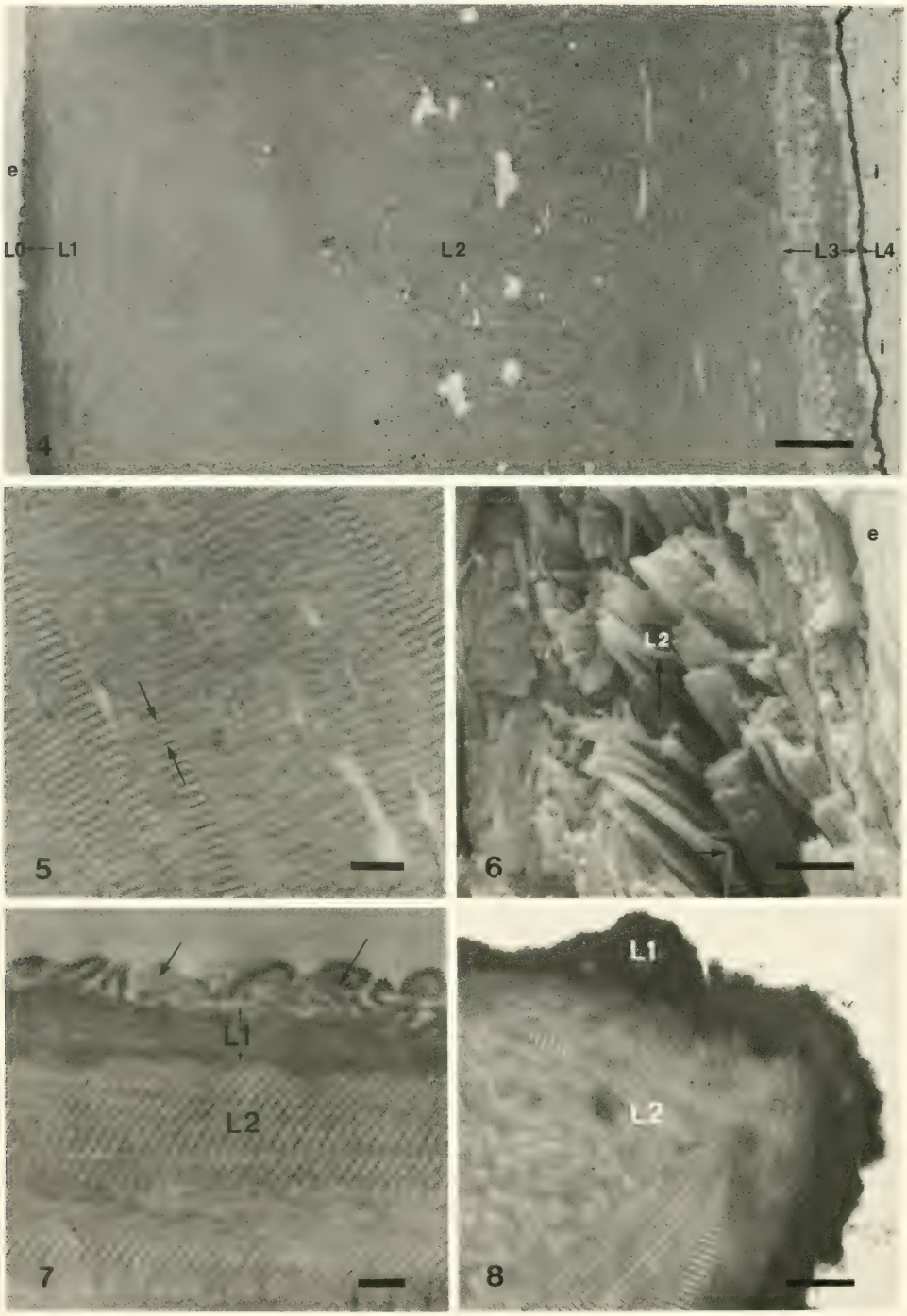


FIGURE 4. Transmission electron micrograph (TEM) of a longitudinal section of the capsule wall from a mature capsule of *Ilyanassa obsoleta*. The locations of the four layers of the capsule (L1–L4) as well as the fibrous surface layer (L0), the capsule exterior (e), and interior (i) are indicated. Bar = 1 μm.

wall 11–12 μm thick and is described in detail below. The surface of a capsule is covered by a fibrous, electron-dense layer (L0) that is not considered part of the capsule wall proper and will be discussed later. Underneath the fibrous layer is the first true wall layer (L1), which is an electron-dense layer surrounding most of the capsule. The material of this layer is quite uniform in thickness (200–250 nm) in mature capsules. Over 80% of the capsule wall (9–10 μm) consists of striated fibers located in the second layer (L2). The striations run perpendicular to the long axis of a fiber and have a periodic pattern with a repeat of 90–100 nm (Fig. 5). Single fibers are usually 60–80 nm in diameter, with the fibers organized into three bundles. Two groups of fibers are visible in longitudinal sections of the capsule, one close to L1 and the other in contact with the adjacent third layer (Fig. 4). The other bundle of fibers occupies the central region of L2 and is seen only in cross sections of a capsule. This layer of the capsule wall appears very homogeneous by scanning electron microscopy when capsules were cut with a razor blade prior to fixation (see Fig. 9). However, ethanol cryofracture of the capsule wall shows the orientation of the bundles of L2 fibers (and some individual fibers) very clearly (Fig. 6). The third layer of the wall (L3) is approximately 1.0 μm thick and consists of two groups of loosely packed 10 nm filaments on either side of a central electron-dense material (Fig. 4). Finally, the innermost portion of the wall (L4) is an electron-dense layer measuring 60 nm in thickness that completely lines the embryo chamber in all regions of the capsule. The dimensions of the layers of the wall must be regarded as approximations due to the variability of the overall size of individual capsules and to an average 5–10% shrinkage of the capsule during specimen preparation.

Capsules collected from the mantle cavity (Fig. 1) and mature capsules (Fig. 3) contain all four capsule wall layers described above, demonstrating that these layers are secreted along the oviduct. In addition, mature capsules are covered by a surface layer that is visible in electron micrographs. In thin sections, this outer layer (L0) is studded with 11–12 nm granules and usually varies from 100 to 200 nm in thickness (Fig. 7), though occasionally it reaches 500 nm in thickness. This surface layer is only present on capsules that have been sculptured in the foot and is absent from capsules collected directly from the mantle cavity (Fig. 8). In scanning electron micrographs of the surface of mature capsules L0 appears as a fibrous network that varies in thickness in different regions of the surface (Fig. 9).

Although all layers of the capsule wall described above extend into the capsule apex, their organization here is more complex. L1 and L2 occupy the outer regions of the apex (Fig. 10), as they do in the wall, but L3 expands in the central region of the apex to form most of the capsule plug (see Fig. 13). The plug is a small disk measuring 100–150 μm in thickness and 400–500 μm in diameter. The 10 nm filaments of L3 are also present in the plug but they appear more compact than they are in the wall and are arranged in a tight meshwork with filaments parallel and perpendicular to the plane of section (Fig. 11). Two other layers of the capsule contribute material

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FIGURE 5. TEM of the fibers of L2 from the *Ilyanassa obsoleta* capsule wall. The 100 nm periodicity of the striations (between the arrows) is repeated along the length of a fiber. Bar = 0.2 μm.

FIGURE 6. Scanning electron micrograph (SEM) of an oblique view of the inside of the *Ilyanassa obsoleta* egg capsule wall following ethanol cryofracture. Individual fibers (arrows) and fiber bundles are visible in several orientations. Bar = 2.0 μm.

FIGURE 7. TEM of the outer region of the mature capsule wall of *Ilyanassa obsoleta* showing the granular surface layer L0 (arrows), L1, and a portion of L2. Bar = 0.2 μm.

FIGURE 8. TEM of the egg capsule of *Ilyanassa obsoleta* wall after a capsule has emerged from the oviduct but before entering the foot (see Fig. 1). L2 is present, but L1 is less uniform in thickness than in mature capsules, and L0 is absent. Bar = 0.4 μm.

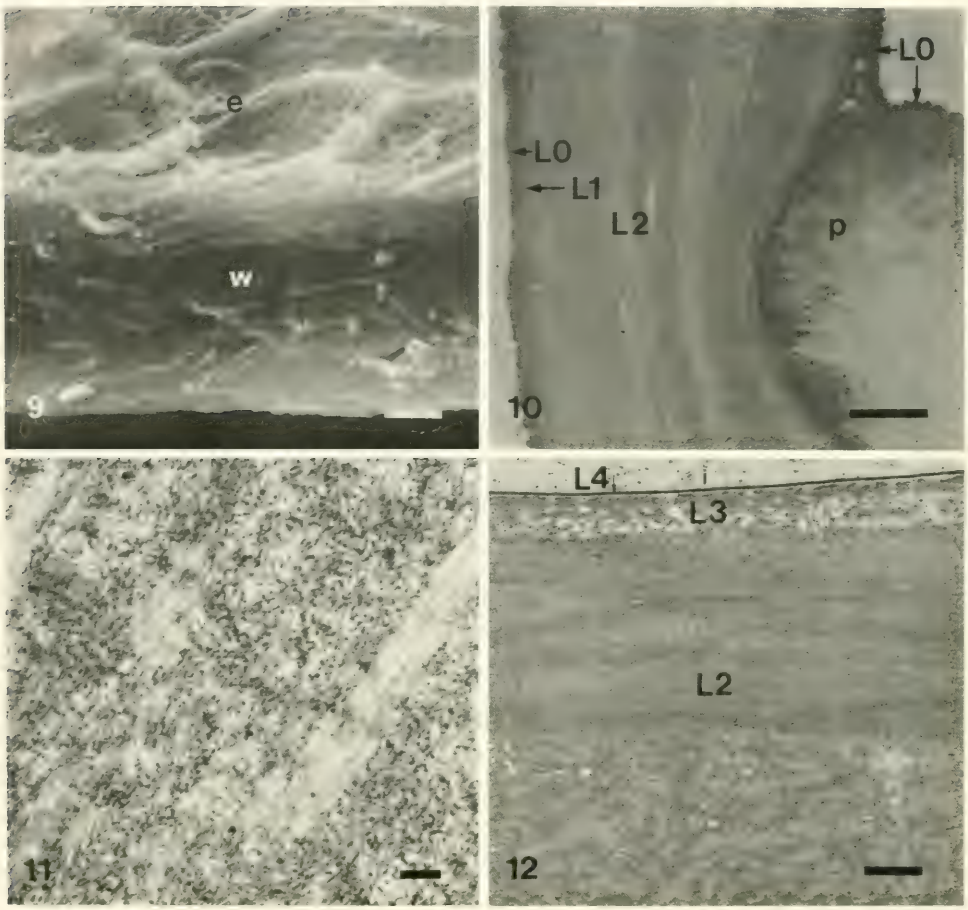

FIGURE 9. SEM of the *Ilyanassa obsoleta* egg capsule wall (w). The capsule was cut along its long axis before fixation to expose the interior of the wall. The exterior of the capsule wall (e) is covered with fibrous material. Bar = 2 μm.

FIGURE 10. TEM of the apical region of the egg capsule of *Ilyanassa obsoleta* in the region of contact between the plug (p) and the capsule wall (see asterisk in Fig. 13 for reference). L0 is visible around the outside of the capsule wall and on top of the plug. The spine adjacent to the plug contains primarily fibers of L2. Bar = 1 μm.

FIGURE 11. High magnification TEM of the capsule plug filaments from a newly deposited capsule of *Ilyanassa obsoleta*. Filaments are visible parallel and perpendicular to the plane of section. Bar = 0.1 μm.

FIGURE 12. TEM of the floor of the embryo chamber of the *Ilyanassa obsoleta* egg capsule (see "f" on Fig. 3 for reference). Starting from the interior of the capsule (i), layers L4, L3, and a portion of L2 are present. Bar = 1 μm.

to the plug. L0 is present over the surface of the capsule wall and plug in the apex (Fig. 10), while L4 is present as the innermost layer of this region.

The floor of the embryo chamber (Fig. 12) contains at least three of the structural layers of the capsule wall and like the wall, most of the floor consists of the fibers of L2. However, because capsules were collected by scraping them off the sides of the aquarium with razor blades, it is difficult to conclude whether L1 is present because L1 (along with a portion of L2) may be left behind explaining why they are not

present in micrographs of this area. Also, because the embryo chamber floor and the basal disk extending from it protrude from the pedal gland, they probably are not covered with the material of L0.

The numerous projections on the capsule surface and the basal disk (Fig. 3) contain primarily the fibers of L2 indicating that this layer is extensively redistributed during the sculpturing of the capsule that occurs in the foot. The material of L1 is also redistributed by ventral pedal gland activity. Before capsules are sculptured, L1 can vary from 100–500 nm in thickness (Fig. 8), but L1 is moulded into a more uniform layer while in the pedal gland (Fig. 7).

Histochemistry

The *Ilyanassa* egg capsule is composed of protein and carbohydrate. With the exception of sudan black, stains utilized in this study were positive for at least some layers of the capsule wall and plug. At the level of light microscopy, L1 could not be differentiated from L0, so that the results for L1 include both layers, and L4 could not be resolved well. Therefore, the histochemical results include only L1, L2, and L3.

Results of staining thick sections of the capsule (Fig. 13) and from staining of the capsule before sectioning (Table I) show that all layers of the *Ilyanassa* capsule wall contain protein, but only L1, L3, and the plug were very positive for aromatic amino

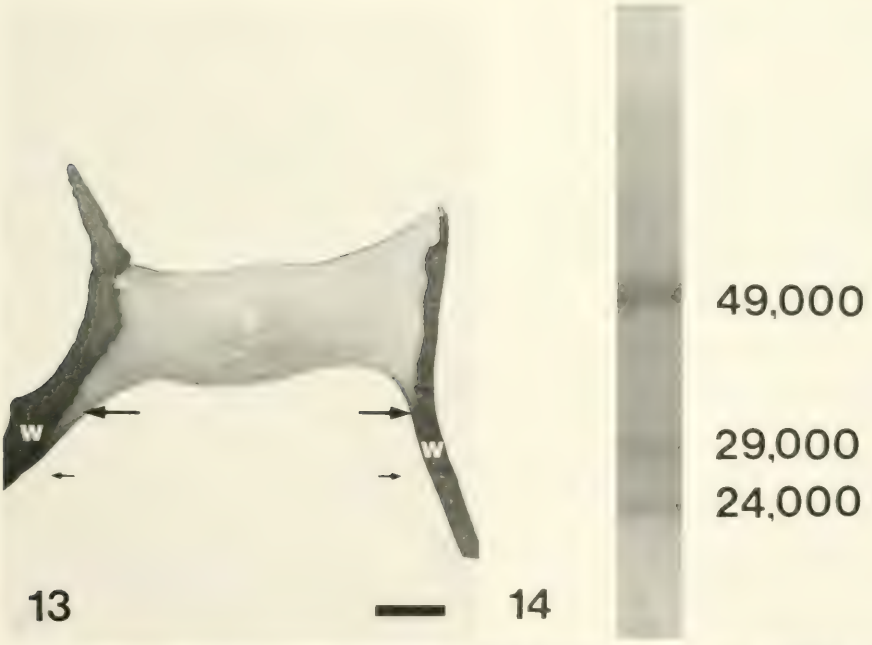

FIGURE 13. Light micrograph of a longitudinal section of the apical region of an *Ilyanassa obsoleta* capsule showing L3 of the capsule wall (large arrows) expanding to form the plug (the lighter region designated p). Some capsule fluid (small arrows) is still present internal to the capsule wall (w). The asterisk identifies the area shown at higher magnification in Figure 10. The capsule has been stained with a polychrome stain (Richardson *et al.*, 1960). Bar = 100 μm.

FIGURE 14. Analysis of the major proteins obtained from the plugs of *Ilyanassa obsoleta* egg capsules. Proteins were separated on a 12.5% polyacrylamide-SDS gel and were stained with Coomassie blue.

acids (Baker's test) and only L1 and L2 were very positive for basic amino acids (fast green). Staining of L3 and the plug with fast green was very faint. The capsule is also composed of carbohydrate. All layers of the capsule were positive with periodic acid-Schiff's stain, but only L3 and the plug stained for acid mucopolysaccharide with alcian blue. The acidic nature of these mucosubstances was due to carboxyl groups, since L3 and the plug were stained with alcian blue containing less than 0.6 *M* $MgCl_2$. Staining at or above 0.8 *M* $MgCl_2$ was very pale, demonstrating that sulfate groups were not abundant. The acidic groups in L1, L3, and the plug were organized in the proper orientation to produce a reddish-purple metachromasia with toluidine blue. Metachromasia, the shifting of the color of toluidine blue from blue to red, is produced when the acidic groups to which the dye binds are arranged closely (less than 5 Å, Pearse, 1968) so that individual dye molecules can interact. L1 and L2, being periodic acid-Schiff's positive and alcian blue negative, were composed of neutral polysaccharides.

Polyacrylamide gel electrophoresis

SDS-polyacrylamide gel electrophoresis of capsule plugs revealed three major glycoproteins with approximate molecular weights of 49,000, 29,000, and 24,000 daltons (Fig. 14). The 49,000 dalton band was the major Coomassie blue positive band while the 29,000 dalton band was the major periodic acid-Schiff's positive band. The specificity of the periodic acid-Schiff's procedure for carbohydrate moieties was verified when ovalbumin was the only molecular weight standard detected by the stain.

DISCUSSION

The arrangement of the *Ilyanassa* capsule into several layers is similar to other egg capsules and, therefore, may be a common feature of gastropod capsule organization (Table II). However, there are differences among gastropods in the arrangement and formation of their egg capsules and minor differences in composition.

Several differences are apparent when comparing the ultrastructure of the capsule of *Ilyanassa* to that of *Urosalpinx,* the only other prosobranch gastropod capsule

TABLE II

Organization of the egg capsule walls of five prosobranch gastropod molluscs

Animal	Outer layer		Inner layer		Authors
Urosalpinx cinerea	L1 (30 μm)	L2 (75 μm)	L3 (2 μm)	L4 (2 μm)	Tamarin and Carriker, 1967
Nucella lapillus	Mucopoly-saccharide layer	L2: Circular fibrous layer; Long. fibrous layer; Outer homogeneous layer	Inner homogeneous layer	Not detected	Bayne, 1968
Busycon carica *B. canaliculatum*	L1	L2: Long. fibrous zone; Circular fibrous zone; Outer homogeneous zone	L3	L4	Harasewych, 1978
Ilyanassa obsoleta	L1 (0.2 μm)	L2: Long. Fibers; Circular Fibers; Long. Fibers (9–10 μm)	L3 (1.0 μm)	L4 (60 nm)	This study

examined in detail by transmission electron microscopy (Tamarin and Carriker, 1967). The outer layer of the *Urosalpinx* capsule is a very diffuse network of striated fibers arranged circularly around the capsule. The striated pattern of these fibers has a periodicity of 53 nm, which Tamarin and Carriker (1967) termed "reminiscent of collagen." These fibers also make up the second layer and are arranged in concentric circular and longitudinal bundles. Occasionally, spaces or "vacuoles" are seen between the fibers of L2. The third layer of the *Urosalpinx* capsule is an aggregation of 5 nm filaments, while L4 contains some of the striated fibers seen in other layers and a thin inner "amorphous," electron-dense layer. In the *Ilyanassa* capsule, however, L1 is electron-dense and compact, with fibrous material (L0) present on the capsule surface external to L1. The striated fibers of the *Ilyanassa* capsule wall are restricted to L2 and are seen in all planes of section as two groups of longitudinal fibers around a central, circular band. The periodicity of the striated pattern of these fibers (90–100 nm) is very similar to the pattern seen in the capsule wall fibers of *Buccinum undatum*, which have a 100–110 nm periodicity in an alpha-helical pattern (Flower *et al.*, 1969) and are termed "keratin-like" (Rudall, 1968). The vacuoles seen between the fibers of L2 in *Urosalpinx* (Tamarin and Carriker, 1967) are occasionally seen as clear areas between the striated fibers in the walls of the *Ilyanassa* capsule (Figs. 4, 5). The size of the filaments in L3 of *Urosalpinx* and *Ilyanassa* is comparable, but in *Urosalpinx* L3 is not continuous with the capsule plug. Rather, the lower portion of the plug of the *Urosalpinx* capsule is composed of the same material found in L2 (Hancock, 1956; Tamarin and Carriker, 1967) while only the outer portion of the plug contains the filaments found in L3. A "chromotropic zone" of diffuse material, which lies around the plug separating it from the capsule walls (Tamarin and Carriker, 1967), may be functioning as a "cementum" holding the plug in place until hatching (Hancock, 1956). Finally, the thin electron-dense layer that makes up all of L4 in *Ilyanassa* capsules is only the innermost portion of a more complex L4 in *Urosalpinx*.

There is no evidence from the arrangement of the four layers of the *Ilyanassa* egg capsule wall that the embryos are inserted into an already secreted capsule as described by Fretter (1941) for the large capsules of *Nucella lapillus*. The relatively small size of the *Ilyanassa* capsule in relation to the size of the capsule gland along the oviduct (about one third the size of the gland) may allow for the sequential secretion of layers of the capsule wall around the mass of embryos that were released prior to capsule formation as occurs during the formation of the capsule around *Nassarius* eggs (Fretter, 1941). The concentric arrangement of the layers of the *Ilyanassa* capsule wall around the embryos supports the latter mechanism: L4 and L3 entirely surround the mass of embryos, while L2 and L1 are present as the outer regions of the capsule except on the plug itself.

There has been discussion whether the ventral pedal gland contributes to capsule formation (Fretter and Graham, 1962). At least four events occur while a capsule is maturing in the ventral pedal gland: sculpturing of the capsule surface by a redistribution of layers L1 and L2; hardening of the capsule wall, possibly by chemical cross-linking (Price and Hunt, 1973, 1976); fibrous material is added onto the capsule surface; and the capsule is attached by its basal disk to some substrate. Our study shows that the four structural layers of the *Ilyanassa* egg capsule wall identified by transmission electron microscopy originate in the oviduct so that the ventral pedal gland does not secrete any of the structural layers of the capsule wall in *Ilyanassa*. However, the layers are modified while a capsule is in the pedal gland. The chemical reactions occurring during hardening of the capsule wall may explain the different electron densities of L1 observed before and after capsules have passed through the gland (compare Figs. 7 and 8).

TABLE III

Comparison of the histochemical composition of the capsules of Nucella lapillus *(Bayne, 1968) and* Ilyanassa obsoleta

	Ilyanassa			Nucella		
Compound	L1	L2	L3	L1	L2	L3
Protein	+	+	+	+	+	+
Basic Amino Acids	+	+	very pale	+	+	+
Tryosine	+	−	+	+	+	0
Carbohydrate	+	+	+	+	+	+
Acid Mucopolysaccharide	−	−	+	+	−	+
Metachromasia	+	−	+	+	−	+
Lipids	−	−	−	−	−	−

+ = Positive.
− = Negative.
0 = Not Tested.

The capsule of only one other prosobranch gastropod has been examined by histochemistry. Bayne (1968) found that the *Nucella* egg capsule is also composed of carbohydrate and protein, with no evidence of lipid being present (Table III). Our results for the *Ilyanassa* capsule are in excellent agreement with those for *Nucella* indicating the capsules of these two prosobranchs have a similar composition as well as a similar organization. The only differences in histochemical composition between the two capsules were the detection of acid mucopolysaccharides in L1 of *Nucella* and minor differences in amino acid composition (Bayne, 1968).

As discussed earlier, many embryos release enzymes in order to hatch from egg envelopes. It is not known whether the hatching substance released by *Ilyanassa* embryos to dissolve the capsule plug is also an enzyme. However, if it is, it must be able to dissolve L4 and L0 as well as the protein and/or carbohydrate components of L3. Protease activity is released from *Ilyanassa* embryos at the time of hatching (Sullivan and Bonar, 1984) and our histochemical tests have identified amino acid groups cleaved by chymotrypsin (aromatic amino acids) and trace amounts of those cleaved by trypsin (basic amino acids). The dual carbohydrate and protein composition of gastropod egg capsules has been measured more directly by chemical analysis (Hunt, 1966). The capsules of *Buccinum* contain 78% protein and only 8% carbohydrate by weight. Our results from gel electrophoresis and histochemistry demonstrate that the plug region of *Ilyanassa* capsules dissolved during hatching also has a dual composition. Our plug samples do contain small portions of L0 and L4, but because most of the plug region is L3, we suspect that all three glycoproteins identified are from the latter layer.

We are currently examining the ability of several proteases to mimic the action of the hatching substance and we are trying to determine if the hatching substance degrades any of the three plug glycoproteins in order to elucidate a mechanism of action of the *Ilyanassa* hatching substance.

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LITERATURE CITED

BAYNE, C. J. 1968. Histochemical studies of the egg capsule of eight gastropod molluscs. *Proc. Malac. Soc. Lond.* **38:** 199–212.

CHAYEN, J., L. BITENSKY, AND R. BUTCHER. 1973. *Practical Histochemistry.* John Wiley & Sons, London. 271 pp.

DAVIS, C. C. 1968. Mechanisms of hatching in aquatic invertebrate eggs. *Oceanog. Mar. Biol. Ann. Rev.* **6:** 325–376.

FAIRBANKS, G., T. STECK, AND D. F. H. WALLACH. 1971. Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. *Biochemistry* **10:** 2606–2617.

FLOWER, N. E., A. J. GEDDES, AND K. M. RUDALL. 1969. Ultrastructure of the fibrous protein from the egg capsules of the whelk *Buccinum undatum*. *J. Ultrastruc. Res.* **26:** 262–273.

FRETTER, V. 1941. The genital ducts of some British stenoglossan prosobranchs. *J. Mar. Biol. Assoc. U.K.* **25:** 173–211.

FRETTER, V., AND A. GRAHAM. 1962. *British Prosobranch Molluscs.* Ray Society, London. 755 pp.

HANCOCK, D. A. 1956. The structure of the capsule and the hatching process in *Urosalpinx cinerea* (Say). *Proc. Zool. Soc. Lond.* **127:** 565–571.

HARASEWYCH, M. G. 1978. Biochemical studies of the hatching process in *Busycon.* Master's Thesis, University of Delaware, Newark. 52 pp.

HUMASON, G. L. 1979. *Animal Tissue Techniques.* 4th Ed. W. H. Freeman and Co., San Francisco, 661 pp.

HUMPHREYS, W. J., B. O. SPURLOCK, AND J. S. JOHNSON. 1974. Critical point drying of ethanol-infiltrated, cryofractured biological specimens for scanning electron microscopy. Pp. 275–282 in *Scanning Electron Microscopy 1974,* O. Johari and I. Corvin, eds. ITT Research Institute, Chicago.

HUNT, S. 1966. Carbohydrate and amino-acid composition of the egg capsule of the whelk *Buccinum undatum* L. *Nature* **210:** 436–437.

LAEMMLI, U. K. 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature* **227:** 680–685.

LUFT, J. H. 1961. Improvements in epoxy resin embedding methods. *J. Biophys. Biochem. Cytol.* **9:** 409–414.

PEARSE, A. G. E. 1968. *Histochemistry, Theoretical and Applied,* 3rd Ed, Vol I. Little, Brown & Company, Boston. 759 pp.

PECHENIK, J. A. 1975. The escape of veligers from the egg capsules of *Nassarius obsoletus* and *Nassarius trivittatus* (Gastropoda, Prosobranchia). *Biol. Bull.* **149:** 580–589.

PRICE, N. R., AND S. HUNT. 1973. Studies of the cross-linking regions of whelk egg-capsule proteins. *Biochem. Soc. Trans.* **1:** 158–159.

PRICE, N. R., AND S. HUNT. 1976. An unusual type of secretory cell in the ventral pedal gland of the gastropod mollusc *Buccinum undatum* L. *Tissue Cell* **8:** 217–228.

RICHARDSON, K. C., L. JARETT, AND E. H. FINKE. 1960. Embedding in epoxy resins for ultrathin sectioning in electron microscopy. *Stain Technol.* **35:** 313–323.

RUDALL, K. M. 1968. Intracellular fibrous proteins and the keratins. Pp. 584–588 in *Comprehensive Biochemistry 26* B, M. Florkin and E. H. Stotz, eds. Elsevier, Amsterdam.

SCHELTEMA, R. S. 1967. The relationship of temperature to the larval development of *Nassarius obsoletus* (Gastropoda). *Biol. Bull.* **132:** 253–265.

STEFFENS, W. L. 1978. A method for the removal of epoxy resins from tissue in preparation for scanning electron microscopy. *J. Microscopy* **113:** 95–99.

SULLIVAN, C. H., AND D. B. BONAR. 1984. Biochemical characterization of the hatching process of *Ilyanassa obsoleta*. *J. Exp. Zool.* **229:** 223–234.

TAMARIN, A., AND M. R. CARRIKER. 1967. The egg capsule of the muricid gastropod *Urosalpinx cinerea:* An integrated study of the wall by ordinary light, polarized light, and electron microscopy. *J. Ultrastruc. Res.* **21:** 26–40.

VAUGHN, C. M. 1953. Effects of temperature on hatching and growth of *Lymnaea stagnalis appressa* Say. *Am. Midl. Nat.* **49:** 214–228.

VENABLE, J. H., AND R. COGGESHALL. 1965. A simplified lead citrate stain for use in electron microscopy. *J. Cell Biol.* **25:** 407–408.

WEBER, K., AND M. OSBORN. 1969. The reliability of molecular weight determinations by dodecyl-sulfate-polyacrylamide gel electrophoresis. *J. Biol. Chem.* **244:** 4406–4412.

Reference: *Biol. Bull.* **167**: 390–409. (October, 1984)

SKELETAL STRENGTH OF ENCRUSTING CHEILOSTOME BRYOZOANS

BARBARA A. BEST[1] AND JUDITH E. WINSTON[2]

[1] *Department of Zoology, Duke University, Durham, North Carolina 27706, and* [2]*Department of Invertebrates, American Museum of Natural History, New York, New York 10024*

ABSTRACT

Encrusting cheilostome bryozoans structurally resemble aggregates of small boxes, with both frontal and vertical walls capable of resisting forces generated by water-borne debris or predators. Both the skeletal strength and design of the walls are important in determining the relative ability of the colony to resist damage. Two mechanical tests, puncture and compression, performed on nine species of tropical bryozoans reveal significant differences in skeletal strength both between species and between the outer and inner regions of colonies. Puncture stresses required to break through the frontal walls of zooids range from 0.8 to 291.0 MNm^{-2} for edge zooids and from 1.1 to 457.4 MNm^{-2} for inner zooids; compressive stresses required to damage the colony range from 4.4 to 16.9 MNm^{-2} for edge regions and 6.5 to 27.2 MNm^{-2} for inner regions. Ecological implications for these differences in skeletal strength are discussed with particular reference to resisting predation. From the mechanical test results, the material properties of shear strength (2.6–90.5 MNm^{-2}) and compressive strength (8.2–110.0 MNm^{-2}) are estimated for the frontal and vertical walls, respectively. Bryozoan wall material appears to be comparable in strength to such biological ceramics as coral, echinoid spine, bivalve shell, and vertebrate bone, but lower in strength than gastropod shell.

INTRODUCTION

The Cheilostomata (with approximately 1000 genera) are the dominant group of bryozoans in Recent seas. Much of their evolutionary success has been explained in terms of the ways in which they have solved the problem of increasing calcification, and thereby the support and protection of soft tissues and feeding organs, without sacrificing the hydrostatic method of lophophore protrusion (Cheetham, 1971; Schopf, 1977). Cheilostomes vary widely in the extent of calcification. In the suborder Anasca, zooidal, basal, and vertical walls are usually calcified, while the frontal wall remains membranous to a greater or lesser extent (though overarching spines or an underlying cryptocyst may provide some protection). In contrast, bryozoans of the apparently polyphyletic suborder Ascophora exhibit varying modes of additional calcification of the frontal wall (Banta and Wass, 1979; reviewed in Hayward and Ryland, 1979), which can be further elaborated on by the development of thickened areas, tuberosities, and pores in various patterns.

Surprisingly, in view of the obvious importance of wall structure in the group, experimental studies of how well the skeleton strengthens or protects the zooids are lacking (Schopf, 1977). The only experimental work thus far relates primarily to the resistance of erect species to bending stress as induced by bottom currents or water-

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borne debris (Schopf *et al.*, 1980; Cheetham and Thomsen, 1981). Although the majority of cheilostome species are primarily or entirely encrusting, there has been no experimental work on skeletal strength of encrusting species, except for two species which were incidentally anlyzed to compare with erect species (Cheetham and Thomsen, 1981).

Zooids of encrusting cheilostomes resemble minature boxes with frontal, vertical, and basal walls surrounding the body cavity. In this study two different mechanical tests, puncture and compression, were performed in an attempt to elucidate the relative strengths of both frontal and vertical walls and their contribution to overall colony protection. Ordinarily, the basal walls of encrusting species are entirely adherent to a rigid substratum and thus not directly involved in strengthening the zooids. Pucture tests were also performed on two structures associated with frontal surfaces, ovicells and opercula. We report here the results of a study of zooid strength in nine species of encrusting cheilostomes, show that differences exist both between species and between inner and outer regions of colonies within a species, and provide some possible structural and ecological interpretations of these differences.

Materials and Methods

Species studied were all from the cryptic coral reef habitat of Jamaica (Jackson and Winston, 1982; Winston and Jackson, 1984). Nine species—*Steginoporella* sp. nov., *Steginoporella magnilabris, Reptadeonella costulata, Reptadeonella bipartita, Trematooecia aviculifera, Parellisina latirostris, Parasmittina* sp., *Stylopoma spongites,* and *Drepanophora tuberculatum* (Fig. 1)—were collected at depths between 10 and 20 meters at two sites, Rio Bueno and Pear Tree Bottom, on the north coast of Jamaica. These species were chosen because they are the most abundant in these environments (Jackson, 1984).

Bryozoan colonies with their coral substrata were collected by diving, transported in sea water to the laboratory, and maintained there in running sea water until tested and measured.

Puncture test

The first mechanical test performed was a "puncture" test, similar to a direct-shear or punching test (Faupel, 1964; Carter *et al.*, 1983), to measure the strength of frontal walls. A puncturing device (Fig. 2A) was constructed by sanding down the point of a straight pin, resulting in a flat blunt circular probe. The probe diameter was measured at 0.115 mm (area = 0.0139 mm^2). The surface area of the probe was measured periodically throughout the series of tests to ensure a constant area.

This puncturing probe was attached to a force platform which could be lowered vertically by a micromanipulator, pushing the probe perpendicularly down onto the surface of a colony, completely submerged in sea water. Viewing through a dissecting microscope allowed for precise placement of the probe on the frontal surface of a zooid, along the mid-line about one half of the way between the proximal wall and the operculum (as shown in Fig. 3F). The platform consisted of two steel beams (0.20 mm thick, 12.7 mm wide) held parallel, restricting bending to only one plane. A strain gauge (Bean, BAE-06-250BB-120TE, 120 ohm) was mounted on one steel beam. As the platform was lowered, the probe pushed against the frontal wall; this force was transmitted along the rod which connected the steel beams, bending both. The strain gauge measured the amount of deflection, or strain, which was recorded as a change in its electrical resistance, amplified by a bridge amplifier, and registered

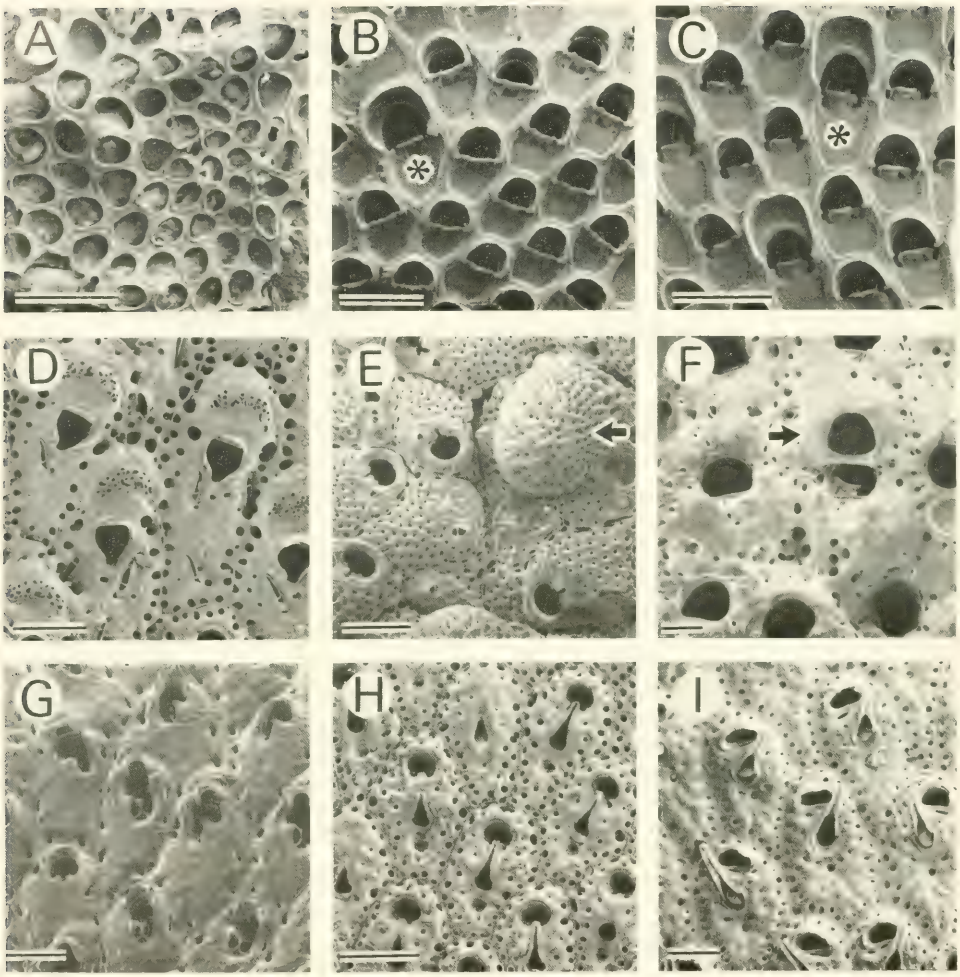

FIGURE 1. Cheilostome species tested showing appearance of frontal surfaces in skeletal (bleached) specimens. A. *Parellisina latirostris* (scale = 1 mm). B. *Steginoporella* sp. nov. (scale = 1 mm). C. *Steginoporella magnilabris* (scale = 1 mm). D. *Parasmittina* sp. (scale = 200 μm). E. *Stylopoma spongites* (scale = 200 μm). F. *Trematooecia aviculifera* (scale = 200 μm). G. *Drepanophora tuberculatum* (scale = 200 μm). H. *Reptadeonella bipartita* (scale = 400 μm). I. *Reptadeonella costulata* (scale = 200 μm). Arrows point to ovicells of *Stylopoma* and *Trematooecia*. Asterisks mark B-zooids in *Steginoporella*.

on a dual channel chart recorder. Once the gauge output is calibrated, this deformation is an indirect measure of the force placed on the colony by the probe.

By inverting the force platform and adding known weights to the probe, the output of the gauge could be calibrated to the nearest 1×10^{-5} N. Calibrations were repeated before and after testing each species. In all the tests, the force platform was lowered at approximately the same rate to ensure nearly uniform loading rates.

The core to a linear variable differential transformer (LVDT Schaevitz 24v, Type 100-HR) was placed along the length of the rod connecting the steel beams to the probe. This transformer recorded the "displacement" (to the nearest 1×10^{-5} mm) of the probe, *i.e.*, the distance the probe traveled as it was forced onto the skeleton,

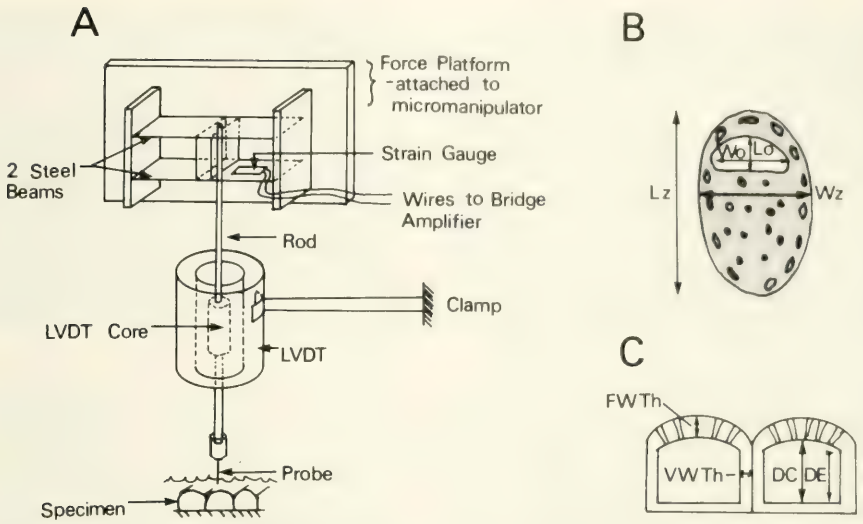

FIGURE 2. A. Diagram of apparatus used to measure skeletal strength of encrusting cheilostome bryozoans. B. Diagram of cheilostome zooid showing standard measurements made on frontal surfaces of zooids. Lz = zooid length. Wz = zooid width. Wo = orifice width. Lo = orifice length. C. Transverse section through two cheilostome zooids, showing other measurements taken. FWTh = frontal wall thickness. VWTh = vertical wall thickness. DC = depth of zooid cavity (center). DE = depth of zooid cavity (edge).

measuring the deformation of the frontal wall before breaking. Displacement registered on the second channel of the chart recorder, resulting in simultaneous reading of both the force applied to and the deformation of the surface.

The forces required to puncture zooecia in the middle and along the growing edge of a colony were recorded for eight species. In addition, both A and B zooids from the inner region of *Steginoporella* sp. nov. were tested. Puncture forces were also recorded for opercula (*e.g.*, Fig. 3A) and ovicells (*e.g.*, Fig. 1E, F) in several species.

Compression test

The same procedures and equipment as in the puncture test were used but with a different probe. A flat end of a drill bit, 0.800 mm in diameter, served as the probe, covering an area 0.503 mm^2. Because zooid size varied between species, the probe spanned between 1 and 5 zooids. Whereas in the puncture test the probe punctured only the frontal wall, in the compression test the vertical walls were primarily involved in resisting the compressive forces of the larger probe.

In the compression tests only growing edge zooids and inner zooids were used. Both regions were tested in six species, inner zooids only in three more.

Morphometrics

After each colony was tested it was measured (live) under the dissecting microscope. For each specimen measurements to the nearest .009 mm were made of length and width of zooids and opercula (Fig. 2B) from both inner and edge regions, and length and width of ovicells if present. Colonies were then broken and examined in side

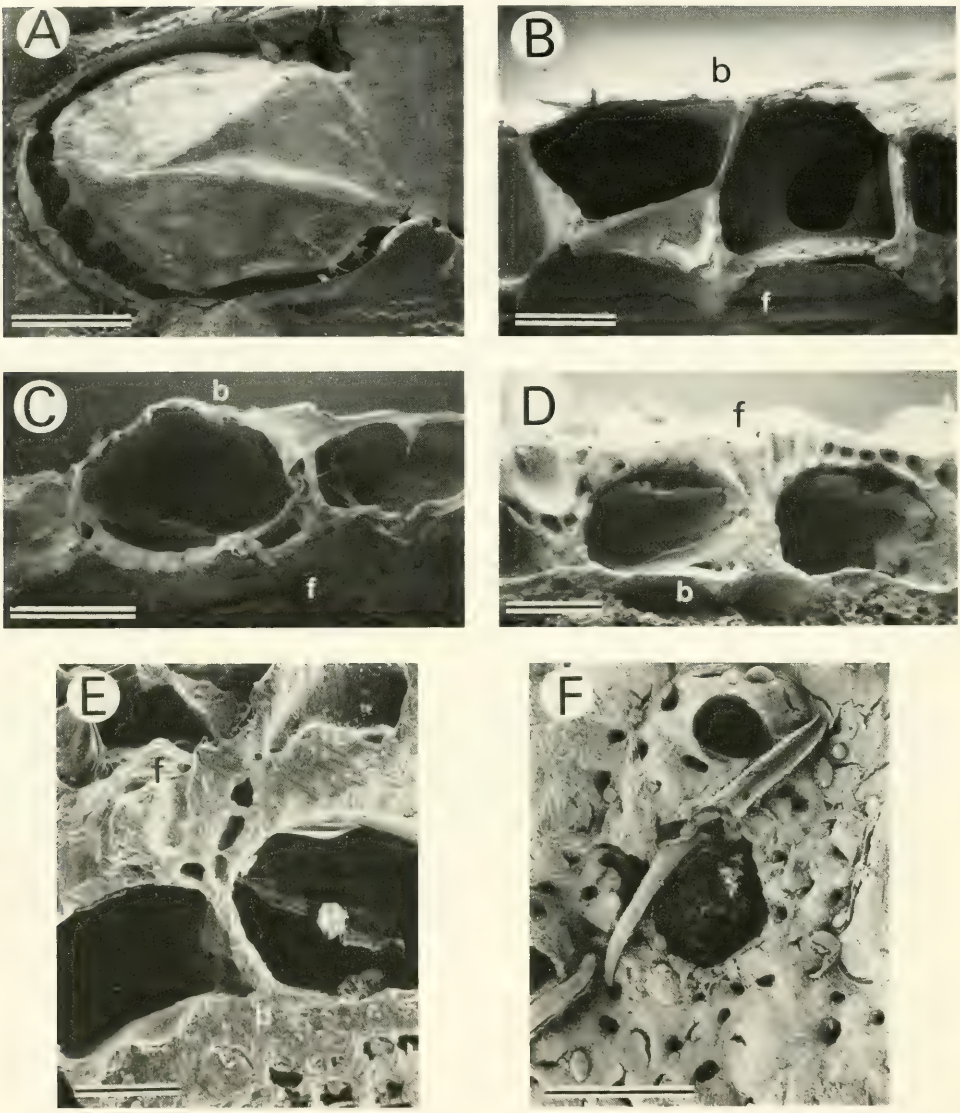

FIGURE 3. All scanning electron micrographs of dried specimens. A. Operculum of B-zooid of *Steginoporella magnilabris* (scale = 100 μm). B. Transverse view of *Steginoporella* sp. nov. zooids (scale = 300 μm). C. Transverse view of *Stylopoma* zooids (scale = 200 μm). D. Transverse view of *Reptadeonella costulata* zooids showing thick vertical and frontal walls (scale = 200 μm). E. Transverse view of *Trematooecia* zooids showing extremely thick frontal walls, but thin vertical walls (scale = 200 μm). F. A-zooid of *Reptadeonella costulata* showing effect of puncture (scale = 200 μm). F = Frontal wall. B = Basal wall.

view to determine frontal wall thickness, vertical wall thickness, and zooid depths at center and edges (Fig. 2C, Fig. 3B–E). Photographs of frontal surfaces of zooids were used to determine both the surface area and perimeter of individual zooids. The length of lateral and transverse walls were measured separately in order to determine the total vertical wall area supporting the zooids under the probe.

Naturally occurring skeletal damage

Additional colonies from Rio Bueno were specially collected, cushioned in plastic bags and very carefully transported to the laboratory to be examined for naturally occurring injuries. For each species tested (except *Parellisina latirostris*) several colonies were examined and types of damage to zooids and larger colony areas noted.

Results

Puncture tests

Zooids. All notations and equations of derived measurements are listed in Appendix 1. Results of the puncture tests of both edge and inner zooids are presented in Appendix 2. Forces (F) applied to zooids were divided by the area of the probe (A_p, 1.039×10^{-8} m^2) to give the applied stress or force per unit area (F/A_p). Values given are the ultimate stress applied before breakage occurred, representing the "puncture strength." The breaking force was also divided by the circumference of the probe (3.616×10^{-4} m) times the frontal wall thickness (T_f) of each species to determine the force per unit area of material in shear ($F/2\pi RT_f$), the "breaking shear stress," which is a material property.

Scheffé's multiple comparison of means procedure (Scheffé, 1959) was used to evaluate significant differences in both puncture strength and shear strength values between species for each edge and inner region (Tables I, II) and within species between the edge and inner regions (Table III).

Table I

Comparison of puncture strength between species for inner and edge zooids

Region/species	Stress (MNm^{-2})
A. Inner zooids	
Parellisina	1.1
Steginoporella magnilabris	7.0
Steginoporella sp. nov. A-zooid	8.0
Steginoporella sp. nov. B-zooid	11.8
Reptadeonella costulata	159.6
Stylopoma	221.0
Drepanophora	286.0
Reptadeonella bipartita	327.3
Trematooecia	457.4
B. Edge zooids	
Parellisina	0.8
Steginoporella megnilabris	2.2
Steginoporella sp. nov. A-zooid	5.0
Stylopoma	33.7
Reptadeonella costulata	67.7
Drepanophora	83.9
Reptadeonella bipartita	126.8
Trematooecia	291.0

Values are mean puncture strengths. Refer to Appendix 2 for sample sizes. There are no statistically significant differences among species within the brackets; all other differences are significant, Scheffé test, $\alpha = 0.05$.

TABLE II

Comparison of shear strength between species for inner and edge zooids

Region/species	Shear stress (MNm^{-2})
A. Inner zooids	
Parellisina	4.8
Steginoporella magnilabris	7.5
Steginoporella sp. nov. A-zooid	9.2
Steginoporella sp. nov. B-zooid	10.6
Reptadeonella costulata	32.8
Drepanophora	75.4
Stylopoma	87.1
Trematooecia	89.5
Reptadeonella bipartita	90.5
B. Edge zooids	
Parellisina	2.6
Steginoporella magnilabris	2.9
Steginoporella sp. nov. A-zooid	6.5
Reptadeonella costulata	17.7
Stylopoma	17.6
Drepanophora	22.1
Reptadeonella	44.5
Trematooecia	83.7

Values are mean shear stresses applied for breakage, *i.e.*, shear strengths. Refer to Appendix 2 for sample sizes. There are no statistically significant differences among species within the brackets; all other differences are significant, Scheffé test, $\alpha = 0.05$.

Opercula. Breaking strengths of opercula were recorded in two ways (Appendix 2). In most cases, the opercular hinge broke as relatively low forces were applied; with increasing loads the probe either broke through the frontal wall of the operculum, or in cases where the probe was larger than the lid, shattered the orifice. A Scheffé test ($\alpha = 0.05$) was used to determine differences in the hinge-breaking stress between species and between A and B zooids of the same species, where applicable.

Opercula of *Steginoporella* sp. nov. A and B zooids were the strongest (4.56 and 6.04 MNm^{-2}), were not different from each other, but were significantly different

TABLE III

Within-species differences in puncture strength and shear strength between edge and inner regions

Species	Puncture strength	Shear strength
Drepanophora	*	*
Parellisina	ns	ns
Reptadeonella bipartita	*	*
Reptadeonella costulata	*	*
Steginoporella magnilabris	*	*
Steginoporella sp. nov. A-zooid	ns	ns
Stylopoma	*	*
Trematooecia	ns	ns

* Significant difference between inner and edge zooids in strength, Scheffé test, $\alpha = 0.05$. ns, not significant.

(Scheffé test, $\alpha = 0.05$) from *Steginoporella magnilabris* (1.59 MNm ^{2}) and *Stylopoma* (2.49 MNm^{-2}). *Trematooecia* (2.73 MNm ^{2}) showed no significant difference from the other species.

Ovicells. Ovicells from four bryozoan species were tested for puncture strengths (Appendix 2). *Drepanophora* (94.59 MNm ^{2}) and *Stylopoma* (70.27 MNm ^{2}) were significantly different (Scheffé test, $\alpha = 0.05$) from *Trematooecia* (22.11 MNm ^{2}). *Parellisina* (52.82 MNm^{-2}) was not different from the others.

Compression tests

The results from the compression tests are listed in Appendix 3. As in the puncture test, breaking forces were converted to force per unit area probe ($A_p = 5.026 \times 10^{-7}$ m^2), or "breaking stress." From the morphometrics a mean value of the total vertical wall area (A_v) under the probe was calculated for each region. This vertical wall area is an estimate of the wall area resisting the compressive force of the probe. Using the mean compressive force for each region, a mean value of force per total vertical wall area (F/A_v) was derived (Appendix 3).

Differences in compressive breaking stress between species were tested for inner and edge regions by a Scheffé test (Table IV). Differences within species between edge and inner regions or between inner A and B zooids are given in Table V.

During the compression tests, observations were made on the nature of breakage for each species. Colonies of *Parasmittina* and *Parellisina* shattered under compression; large cracks radiated outward from the area under stress. *Trematooecia* shattered downward rather than laterally, pulverizing the underlying zooids but not the neigh-

TABLE IV

Comparison of compressive strength between species for inner and edge regions

Region/species	Compressive strength (MNm^{-2})
A. Inner region	
Parellisina	6.5
Steginoporella magnilabris A-zooid	11.3
Stylopoma	12.9
Trematooecia	13.3
Steginoporella magnilabris B-zooid	14.7
Steginoporella sp. nov. A-zooid	14.9
Parasmittina	15.8
Reptadeonella bipartita	23.8
Reptadeonella costulata	26.8
Drepanophora	27.2
B. Edge region	
Steginoporella sp. nov. A-zooid	4.4
Stylopoma	6.3
Trematooecia	7.0
Reptadeonella bipartita	12.6
Reptadeonella costulata	14.3
Drepanophora	16.9

Values are mean compressive strengths. Refer to Appendix 3 for sample sizes. There are no statistically significant differences among species wihtin the brackets; all other differences are significant. Scheffé test, $\alpha = 0.05$.

TABLE V

Within species differences in compressive strength between edge and inner regions or between A- and B-zooids

Species	Regions	Compressive strength
Drepanophora	Edge *vs.* Inner	*
Reptadeonella bipartita	Edge *vs.* Inner	ns
Reptadeonella costulata	Edge *vs.* Inner	*
Steginoporella magnilabris	A- *vs.* B-zooids	ns
Steginoporella sp. nov.	Edge *vs.* Inner	*
Stylopoma	Edge *vs.* Inner	*
Trematooecia	Edge *vs.* Inner	*

* Significant difference between regions in strength, Scheffé test, $\alpha = 0.05$. ns, not significant.

boring ones. These three species had the smallest displacement, or downward movement of the probe, before breakage (Appendix 3). *Parasmittina* and *Parellisina* can be characterized as "brittle" material; during compression the material undergoes little deformation until the breaking stress is reached and then shatters explosively as energy is released. Propogating cracks resulted in more extensive damage to the colony. Shattering in the "rigid" *Trematooecia,* with its heavily calcified frontal walls, occurred when the applied load buckled the thinner vertical walls (Fig. 3E). In the other six species damage was localized and restricted to the area under the probe or immediately adjacent; higher displacements recorded in this group resulted from the vertical walls crushing rather than buckling.

Morphometrics

Direct measurements on zooecial characteristics are presented in Appendix 4 (for all species except *Parasmittina* sp.). These measurements were then used to calculate derived measurements (refer to Appendix 1): number of zooids per probe area (Z_a), total vertical wall area per probe area (A_v), and curvature of the frontal wall (C). An estimate of the frontal wall curvature (C) was derived by subtracting the depth of the zooid cavity edge (DE) from the depth of the zooid cavity at the center (DC), and dividing by the zooid width (W_z). Correlations between both direct and derived measurements were run against compressive breaking force (Table VI). For the puncture test results, the mean displacement of the probe for each species was negatively

TABLE VI

Correlations of structural parameters with compressive breaking force

Parameter	*r*	Prob > *r*	# Obs.	Region
# Zooids/probe area, (Z_a)	0.399	0.286	9	inner
Vertical wall thickness, (T_v)	0.673	0.067	9	inner
Frontal wall thickness, (T_f)	0.678	0.065	8	inner
Total vertical wall area, (A_v)	0.833	0.010*	8	inner
A_v x Puncture shear strength	0.853	0.008**	8	inner
	0.960	0.002**	6	edge

* Denotes $P < 0.05$. ** indicates $P < 0.01$. # Obs. refers to the number of species used in the correlation.

correlated with increasing curvature of the frontal wall ($r = -0.69$, $P = 0.039$) and with the mean shear strength ($r = -0.81$, $P = 0.007$).

Naturally occurring skeletal damage

No new injuries could be detected in the one colony of *Parellisina latirostris* obtained except where zooids had been punctured.

The species showing the greatest amount of frontal wall injury was *Stylopoma spongites*. Many of these injuries consisted of fractures extending over the surfaces of several zooids, or large irregularly shaped patches on the frontal wall of a single zooid.

In colonies of *Reptadeonella costulata* zooids and buds around the margin showed signs of pruning by grazers. The most common injury, however, consisted of abrasion of the outer cuticle and epithelium from part or all of the frontal surface. One zooid had a puncture through the avicularium-spiramen area in the center of the frontal wall. Other colonies were attacked or colonized from beneath by boring sponges and spionid-like polychaetes. Injuries to *Reptadeonella bipartita* followed a similar pattern with some injuries caused from below by the activities of sponges and polychaetes and some by frontal abrasion.

In the most common type of injury to a single zooid of *Steginoporella* sp. nov. the operculum was missing but the skeleton was undamaged; the tissue was gone and there was often a copepod in the zooid interior. In addition to single zooid injuries, "bite-marks" covering groups of zooids (several mm^2 in area) were also noted; there were even larger grazed areas present in old areas of colonies and along the growing margin.

Colony margins of *Drepanophora tuberculatum* showed signs of pruning by grazers, while zooids in the inner regions appeared undamaged except for abrasion of the frontal surface. No injuries were observed on the colonies of *Trematooecia aviculifera* examined, though old injuries may have been obscured by frontal budding.

Discussion

Studies of bryozoan ecology have shown that certain types of predators are common to most ecosystems: single zooid predators—some nudibranchs and pycnogonids—and grazers—urchins, nudibranchs, fish (Ryland, 1976; Seed, 1976; Nybakken and McDonald, 1981; Todd, 1981; Yoshioka, 1982). Another factor in bryozoan ecology is the potential for physical abrasion due to water-transported particles or rubble. Both inner and outer regions of colonies were tested for skeletal strength because it seemed obvious that zooids of the growing edge, in which calcification or development could still be incomplete, might be more susceptible to damage.

Damage to colonies in the field may occur under a variety of loading situations including shear, compression, abrasion, and complex combinations of these. In order to analyze the *relative* strength of bryozoans, we have made several simplifying assumptions: (1) That zooids can be modeled as little boxes, with forces being resisted primarily by the frontal and vertical walls. This assumption neglects the role that additional struts may play in shoring up the skeleton. (2) That the pure puncture or compression tests performed model the action of either impacting debris or a predator. Nudibranch radulas tend to exert a rasping, abrading, or shear action, while grazing, like that of urchins and fish, involve both shear and compression. We assume here

that "rasping" resistance is correlated with "shear" strength and that "grazing" resistance is correlated with "compressive" strength, and thus our tests give a relative measure of "resistance to predation." It is know that the "hardness," as measured by the Vickers hardness number, of a crystalline material is not only indicative of the material's resistance to abrasion, but also is correlated with the shear strength and tensile strength of the material (refer to Vincent, 1982). However, it must be noted that in rasping and grazing the exact geometry of the cutting edges employed by predators significantly affects their effectiveness. Therefore, in order to study the ability of a group of organisms to resist one particular predator, it would be necessary to replicate the exact morphology of the mandibles or radulas and their cutting motion.

Puncture tests

Puncture strength. The puncture stress is the force per area that would be needed by the potential predator to break into the bryozoan zooid through the frontal wall. Although there are obviously many different factors involved in preying on bryozoans, puncture strength may be one determinant. Most nudibranchs that feed on bryozoans seem to prefer those with mostly membranous frontal walls, but at least some species attack well calcified ascophorans (Ryland, 1976). There is some evidence that nudibranchs which do feed on well-calcified forms may be more specialized in diet than those that prey on membranous forms, perhaps possessing radulas that can mechanically produce the forces necessary to puncture calcified walls (Nybakken and McDonald, 1981).

Parellisina, the only species with a membranous frontal wall, was by far the weakest with respect to puncture strength. Next strongest were both A and B zooids of *Steginoporella* sp. nov. and *Steginoporella magnilabris.* None of these species showed evidence of puncture wounds in the field, but in all of them soft tissue was apparently removed from the frontal surface by abrasion without evidence of puncture. *Stylopoma, Reptadeonella costulata,* and *Drepanophora* were intermediate with regards to puncture strength. Two of these species, *Reptadeonella costulata* and *Stylopoma,* showed evidence of pucture damage to individual zooids in specimens surveyed. *Trematooecia* and *Reptadeonella bipartita,* both of which showed no such damage, were the strongest overall.

In five out of eight species edge zone zooids were significantly weaker (half of them were only half as strong) than inner zooids, suggesting that some species may have regions more vulnerable to damage. This comparative weakness may be due to the rate and nature of the mineralization process during development.

Puncture shear strength. Puncture shear strength ($F/2\pi RT_f$) is a parameter of the frontal wall material, independent of thickness, and thus can be used to compare the material properties of the species tested. Differences in shear strength can be due to variations in ultrastructure and in both the mineral and organic composition. Closely related species such as *Steginoporella magnilabris* and both A and B zooids of *Steginoporella* sp. nov. had similar shear strengths. The two *Reptadeonella* species, on the other hand, showed about a three-fold difference in strength. As *Reptadeonella* was instituted as a convenience genus for all encrusting adenoid species (Harmer, 1957), this difference may signify that the two species are not congeneric, but more distantly related, a possibility supported by some other morphological features (Winston, unpub. data).

With regard to edge *versus* inner zooids the pucture shear strength showed exactly the same trends as puncture strength. Thus, the differences in puncture strength seen between the two regions are not attributable to differences in the thickness of the frontal wall (which varies with age), but indicate a difference in the ultrastructure itself, perhaps due to the development of the mineral-organic matrix. This regional difference in material strength in the encrusting bryozoan species studied here contrasts with work on erect species by Cheetham and Thomsen (1981); in the arborescent bryozoans they studied, there is no difference in material bending strength between thinly and thickly calcified parts of the colony.

In the puncture tests, there was a negative correlation between probe displacement and both shear strength and frontal wall curvature. It appears that a higher shear strength and greater curvature results in a more rigid zooecium, undergoing less deformation for a given force. The work (which equals force times displacement) required for puncture is higher in these more rigid forms.

Opercula strength. The force required to break opercular hinges is relatively low; it is not known how much force would be required to remove the operculum entirely (which may be the important parameter to some predators). Several nudibranchs known to attack the opercular area all appear to remove the operculum by sucking it open. This mode of feeding is more common on prey with membranous frontal walls, although a Ghanaian species of *Corambe* (Mollusca) does feed on a *Trematooecia* species in this manner (Ryland, 1976).

Although in two species the orifice shattered at forces lower than those required to break the frontal walls, injuries surveyed showed no tendency for predators to attack the orifices. There may be some other reasons why predators would be unlikely to attack opercula. Nudibranchs feed by puncturing with the radula, forming a seal around the puncture with the mouth, and then sucking out the zooid contents. Nudibranchs may not be able to form a good seal around puncture holes in the operculum area, perhaps due to shattering during breakage.

Ovicell strength. Ovicells in three of the four species tested were significantly lower in strength than the frontal walls. These three species, *Stylopoma, Drepanophora,* and *Trematooecia* have among the most robust inner zooids. Interestingly the species with the weakest frontal wall, *Parellisina* (1.14 MNm^{-2}) had a much stronger ovicell (52.8 MNm^{-2}), perhaps to prevent damage to, or discourage predation on, the enclosed egg.

Compression tests

Compressive strength, the force per probe area, is a measure of the force required to induce damage to several zooids in a colony. In this test the vertical walls supply most of the resistive force, rather than the frontal walls which supply most of the resistive force in puncture tests. Colony injury could be induced by predators biting or grazing an area of zooids as well as by rubble striking the colony.

The two *Reptadeonella* species and *Drepanophora* were the species with the highest compressive strengths. The survey showed that they were subjected to a large amount of abrasion in which the surface layer of tissue was removed, but the underlying skeleton remained intact, and had colony margins which were heavily grazed. Previous studies at Rio Bueno (Winston and Jackson, 1984) had shown that these three species were the ones most able to survive intense damselfish grazing at one panel site. The other species which also had a high survival rate at this site was *Parasmittina* sp.,

the next strongest species according to the compression test (although significantly below the top three). All other species showed lower, relatively similar compressive strengths, with *Parellisina* being the weakest.

Thus, it appears that the susceptibility or resistance of a species to damage induced by physical factors or particular predators may be explained, as well as predicted, by its skeletal strength. The edge zones of the three strongest species were as strong as the inner zooids of the other species; this suggests that skeletons of similar or weaker strength, which include the entire colony of the weaker species, would be susceptible to predation. Distributions, as well as abundances, of bryozoans may be heavily influenced by their skeletal strengths.

Correlation with colony morphology. Compression tests were designed to measure the relative contribution of vertical walls to resisting force applied to the colony. The thickness of the frontal wall was not significantly correlated with compressive strength (Table VI), suggesting that the vertical walls contribute more than the frontal wall under this kind of test. Compressive strength was also not correlated with the number of zooids per probe area, indicating that having numerous small zooids does not necessarily lead to increased strength. However, total vertical wall area (A_v) was significantly correlated ($r = 0.83$, $P = 0.01$) with compressive strength. For example, of the three strongest species, one, *Drepanophora,* had the smallest zooids of the species tested and the others had intermediate sized zooids.

As a first order approximation it seemed reasonable to assume that material properties of both frontal and vertical walls would be approximately of the same value, although there are often ultrastructural differences between the two areas. We derived a new parameter with units of force using puncture shear stress times the total vertical wall area. This parameter was highly correlated with compressive strength ($r = 0.85$, $P = 0.008$ inner region; $r = 0.96$, $P = 0.002$ edge region), indicating that the differences in compressive strength can be explained by considering both the vertical wall area resisting that force and some measure of the wall's material strength.

Comparative material strength

In this study we were able to measure one material property, that of shear strength, and to derive an estimate of another, compressive strength, by estimating the vertical wall area under stress. The shear strength and compressive strength of various other natural and man-made materials are listed in Table VII as a comparison of the range of values these material properties assume.

Most of the bryozoan wall material studied can be classified as "biological ceramics," substances composed of a crystalline material, such as a calcium salt, with an organic matrix. Both the ratio of organic to mineral content and the nature of the crystalline structure may influence the material's strength. Limestone, a natural ceramic, and concrete, a man-made ceramic, lack any organic matrix and exhibit strengths below that of some bryozoans (Table VII). The compressive strength of concrete can be increased dramatically by the inclusion of a small percent of the polymer polymethylmethacrylate. This polymer is thought to act similarly to an organic matrix by bonding the constituents more tightly, thereby decreasing stress concentrations (refer to Vincent, 1982).

Although the presence of a small amount of organic material may strengthen a ceramic, strength may decrease if the percent of organics becomes too high. One of the more important determinants of tensile strength in vertebrate bone is the percent of mineral (Currey, 1969); the higher the mineral content, the stronger the material.

TABLE VII

Comparison of shear strength and compressive strength of bryozoans with other natural and man-made materials

Material	Shear strength (MNm^{-2})	Compressive strength (MNm^{-2})
Bryozoans	2.9–90.5	8.2–110.0[1]
Cedar[2]	6.1	35.9
Coral skeleton[3]	—	12–81
Gastropod shell[4]	—	196–353
Bivalve shell[4]	—	82–419
Echinoid spine[5]	—	48–72
Horse femur[6]	97	142
Concrete[7]	—	6.9–62.1
Limestone[7]	6.2–15.9	17.9–144.8
Porcelain[7]	—	689.6

[1] Per vertical wall area, not probe area.
[2] Eshbach and Sounders, 1975.
[3] Chamberlain, 1978.
[4] Currey, 1976.
[5] Weber *et al.* 1969.
[6] Adapated from Yamada, 1970.
[7] Mantell, 1958.

Bryozoan wall compressive strength shows a range of values comparable to other biological ceramics—such as coral, bivalve shell, echinoid spine, and vertebrate bone—but appears to be lower than gastropod shell (Table VII). Different structural types of shell material—*e.g.*, nacre, crossed-lamellar, foliated—in molluscs have different compressive and tensile strengths (Currey, 1976). In contrast, Cheetham and Thomsen (1981) in their study of aborescent bryozoan skeletons found that the modulus of rupture, a property used to compare strength in bending, was not related to either skeletal composition (calcite *vs.* aragonite) or microstructure (lamellar *vs.* spherulitic). In this study we did not examine the microstructure of the walls tested, and thus can not draw any inferences between ultrastructure and strength. However, future research might examine the possible relationship between the percent of organics and strength in bryozoan skeletons. In this study the only species with an uncalcified, membranous frontal wall had an extremely low value for wall shear strength.

In summary this study has found that species-specific differences exist in the stresses required to either puncture individual zooids or to crush parts of a colony. These differences appear to be due to both zooid architecture and material properties of the walls. Colony compressive strength may be a useful indicator of "resistance to predation" from grazers, and may be a determinant of species abundances and distributions in areas subjected to heavy predation.

Two measurements of material properties—shear strength of frontal walls and compressive strength of vertical walls—also show species-specific differences, as well as significant within-colony differences. These material properties span a wide range of values, and may be indicative of ultrastructural differences in the organic-mineral matrix.

Bryozoan wall material appears to be stronger than some natural ceramics, comparable in strength to such biological ceramics as coral, echinoid spine, bivalve shell, and vertebrate bone, but lower in strength than gastropod shell.

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LITERATURE CITED

BANTA, W. C., AND R. E. WASS. 1979. Cantenicellid cheilostome Bryozoa: I. Frontal walls. *Aust. J. Zool. Suppl. Ser.* **68:** 1–70.

CARTER, J. M., S. E. SORENSEN, R. R. JOHNSON, R. L. TEITELBAUM, AND M. S. LEVINE. 1983. Punch shear testing of extracted vital and endodontically treated teeth. *J. Biomechanics* **16:** 841–849.

CHAMBERLAIN, J. A., JR. 1978. Mechanical properties of coral skeleton: compressive strength and its adaptive significance. *Paleobiology* **4:** 419–435.

CHEETHAM, A. H. 1971. Functional morphology and biofacies distribution of cheilostome Bryozoa in the Danian Stage (Paleocene) of southern Scandanavia. *Smithson. Contrib. Paleobiol.* **6:** 1–87.

CHEETHAM, A. H., AND E. THOMSEN. 1981. Functional morphology of arborescent animals: strength and design of cheilostome bryozoan skeletons. *Paleobiology* **7:** 355–383.

CURREY, J. D. 1976. Further studies on the mechanical properties of mollusc shell material. *J. Zool.* **180:** 445–453.

CURREY, J. D. 1969. The mechanical consequences of variation in the mineral content of bone. *J. Biomechanics* **2:** 1–12.

ESHBACH, O. W., AND M. SOUNDERS, eds. 1975. *Handbook of Engineering Fundamentals.* John Wiley and Sons, NY. 1562 pp.

FAUPEL, J. H. 1964. *Engineering Design.* John Wiley and Sons, NY. 980 pp.

HARMER, S. F. 1957. The Polyzoa of the Siboga expedition. Cheilostomata Ascophora II. *Siboga Expeditie* **28**(4): 641–1147.

HAYWARD, P. J., AND J. S. RYLAND. 1979. *British Ascophoran Bryozoans.* Academic Press, London. 312 pp.

JACKSON, J. B. C. 1984. Ecology of cryptic coral reef communities. III. Abundance and aggregation of encrusting organisms with particular reference to cheilostome bryozoa. *J. Exp. Mar. Biol. Ecol.* **75:** 37–57.

JACKSON, J. B. C., AND J. WINSTON. 1982. Ecology of cryptic coral reef communities. I. Distribution and abundance of major groups. *J. Exp. Mar. Biol. Ecol.* **57:** 135–147.

MANTELL, C. L. 1958. *Engineering Materials Handbook.* McGraw-Hill, NY. 1959 pp.

NYBAKKEN, J., AND G. MCDONALD. 1981. Feeding mechanisms of west American nudibranchs feeding on Bryozoa, Cnidaria and Ascidiacea, with special respect to the radula. *Malacologia* **202:** 439–449.

RYLAND, J. S. 1976. Physiology and ecology of marine bryozoans. *Adv. Mar. Biol.* **14:** 285–443.

SCHEFFÉ, H. 1959. *The Analysis of Variance.* John Wiley and Sons, NY. 475 pp.

SCHOPF, T. J. M. 1977. Patterns and themes of evolution among the Bryozoa. Pp. 159–207 in *Patterns of Evolution,* A. Hallam, ed. Elsevier Publishing Co., Amsterdam.

SCHOPF, T. J. M., K. O. COLLIER, AND B. O. BACH. 1980. Relation of the morphology of stick-like bryozoans at Friday Harbor, Washington, to bottom currents, suspended matter and depth. *Paleobiology* **6:** 466–476.

SEED, R. 1976. Observations on the ecology of *Membranipora* (Bryozoa) and a major predator *Doridella steinbergae* (Nudibranchiata) along the fronds of *Laminaria saccharina* at Friday Harbor, Washington. *J. Exp. Mar. Biol. Ecol.* **24:** 1–17.

TODD, C. 1981. The ecology of nudibranch molluscs. *Oceanogr. Mar. Biol. Annu. Rev.* **19:** 141–234.

VINCENT, J. F. V. 1982. *Structural Biomaterials.* John Wiley and Sons, NY. 207 pp.

WEBER, J., R. GREER, B. VOIGHT, E. WHITE, AND R. ROY. 1969. Unusual strength properties of echinoderm calcite related to structure. *J. Ultrastruct. Res.* **26:** 355–366.

WINSTON, J. E., AND J. B. C. JACKSON. 1984. Ecology of cryptic coral reef communities. IV. Community development and life histories of encrusting cheilostome Bryozoa. *J. Exp. Mar. Biol. Ecol.* **76:** 1–21.

YAMADA, H. 1970. *Strength of Biological Materials.* Williams and Wilkins, Baltimore. 433 pp.

YOSHIOKA, P. M. 1982. Role of planktonic and benthic factors in the population dynamics of the bryozoan *Membranipora membranacea. Ecology* **63:** 457–468.

APPENDIX I

Notation and derived measurements

A	Area
A_p	Cross-sectional area of probe
A_v	Total vertical wall area per probe area
A_z	Zooid area
C	Frontal wall curvature, $(DC - DE)/W_z$
DC	Depth of zooid cavity (center)
DE	Depth of zooid cavity (edge)
F	Force
L_l	Length of lateral wall
L_o	Orifice length
L_t	Length of transverse wall
L_z	Zooid length
N	Newton
P_z	Zooid perimeter (length of vertical walls)
R	Radius
	Stress, F/A_p
	Shear stress, $F/2\pi RT_f$
T_f	Frontal wall thickness
T_v	Vertical wall thickness
W_o	Orifice width
W_z	Zooid width
Z_a	Number of zooids per probe area, A_p/A_z

APPENDIX 2

Puncture tests

Species	Region	Stress Mean (MNm^{-2})	Stress S.D.	Shear stress Mean (MNm^{-2})	Shear stress S.D.	Displacement Mean (MNm^{-4})	Displacement S.D.	Sample size
Drepanophora tuberculatum	Edge	83.95	56.84	22.15	14.99	0.89	0.31	8
	Inner	285.98	96.51	75.45	25.46	1.36	1.19	10
	Ovicell	94.59	29.95	—	—	—	—	7
Parellisina latirostris	Edge	0.81	0.43	2.59*	1.38	0.94	0.21	10
	Inner	1.49	0.64	4.76	2.05	1.29	0.34	10
	Ovicell	52.82	45.35	—	—	—	—	2
Reptadeonella bipartita	Edge	126.77	54.03	44.46	18.95	0.63	0.62	10
	Inner	327.29	36.14	90.51	9.99	0.62	0.25	10
	Operculum opening	423.69	55.16	—	—	—	—	10
Reptadeonella costulata	Edge	67.72	33.27	15.71	7.71	1.81	0.67	12
	Inner	159.56	40.31	32.78	8.28	1.01	—	10
Steginoporella magnilabris	Edge	22.22	1.11	2.90	1.46	0.99	0.32	10
	Inner	7.05	2.52	7.51	2.68	1.16	0.32	10
	Operculum hinge	1.59	0.96	—	—	—	—	9
Steginoporella sp. nov. A-zooids								
	Edge	4.99	1.39	6.52	1.82	3.11	0.47	11
	Inner	7.98	2.69	9.18	3.09	1.75	0.24	12
	Operculum hinge	4.56	1.31	—	—	—	—	11
B-zooids								
	Inner	11.76	2.64	10.57	2.37	1.79	0.41	10
	Operculum hinge	6.04	1.96	—	—	—	—	10
Stylopoma spongites	Edge	33.67	18.24	17.61	9.54	0.63	0.35	10
	Inner	221.05	38.82	87.09	15.29	0.05	0.02	11
	Operculum hinge	2.49	1.41	—	—	—	—	11
	Operculum opening	111.98	32.86	—	—	—	—	11
	Ovicell wall	70.27	18.98	—	—	—	—	8
Trematooecia aviculifera	Edge	290.98	133.29	83.68	38.33	0.23	—	10
	Inner	457.37	114.39	89.48	22.38	0.06	0.05	11
	Operculum hinge	2.73	1.47	—	—	—	—	2
	Operculum opening	374.83	61.13	—	—	—	—	10
	Ovicell membrane		5.65	—	—	—	—	8
	Ovicell wall	5.07	10.03	—	—	—	—	8
		22.11						

* Based on thickness of inner zooids.

Stress = $F/A = Force/\pi R^2$.

Shear stress = $F/2\pi RT_f$, T_f is frontal wall thickness.

APPENDIX 3

Compression tests

Species	Region	Stress = F/A Mean (MNm^{-2})	Stress = F/A S.D. (MNm^{-2})	Displacement Mean (MNm^{-4})	Displacement S.D. (MNm^{-4})	Force/Lateral Wall Area Mean	Sample Size
Drepanophora tuberculatum	Edge	16.89	2.06	2.64	0.41	18.42	8
	Inner	27.17	6.05	2.24	0.59	33.80	10
Parasmittina sp.	Inner	15.81	5.28	1.42	0.44	45.03	7
Parellisina latirostris	Inner	6.54	3.98	0.83	0.19	—	—
Reptadeonella bipartita	Edge	12.57	3.58	1.12	0.27	8.22	4
	Inner	23.83	8.38	1.93	0.35	29.28	10
Reptadeonella costulata	Edge	14.29	9.18	3.03	1.10	17.95	13
	Inner	26.78	2.71	2.26	0.45	17.53	10
Steginoporella magnilabris	A-zooids Inner	11.33	2.48	1.42	0.52	71.17	10
	B-zooids Inner	14.66	4.24	1.93	0.64	—	10
Steginoporella sp. nov.	Edge	4.47	2.01	2.75	0.73	54.51	10
	Inner	14.90	2.43	3.52	0.90	110.13	10
Stylopoma spongites	Edge	6.27	3.36	0.61	0.11	17.12	10
	Inner	12.97	4.42	1.72	0.55	31.49	12
Trematooecia aviculifera	Edge	6.98	3.18	0.98	0.32	14.86	10
	Inner	13.35	3.58	0.87	0.35	22.90	11

APPENDIX 4

Morphometrics of encrusting bryozoans studied

Species: Region	Means (mm)																					
	Zooid				Orifice				Wall thickness				Cavity depth				Ovicell					
	Length	S.D.	Width	S.D.	Length	S.D.	Width	S.D.	Frontal	S.D.	Lateral	S.D.	Center	S.D.	Edge	S.D.	Length	S.D.	Width	S.D.	Height	S.D.
Drepanophora tuberculatum																						
Edge zooids	.382	.025	.286	.026	.102	.026	.086	.008	.109	.017	.037	.007	.111	.024	.066	.014	.116	.012	—	—	.137	—
Inner zooids	.426	.031	.331	.012	.126	.012	.076	.008														
Parellisina latirostris																						
Edge zooids	.424	.055	.400	.028	.064	.006	.144	.012	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Inner zooids + OV	.506	.023	.391	.050	.089	.004	.154	.021	.009*	—	.008	.003	.116	.024	.086	.018	—	—	—	—	—	—
Reptadeonella costulata																						
Autozooids:																						
Edge	.732	.086	.437	.049	.109	.006	.151	.091	.124	.020	.054	.009	.224	.028	.164	.035	—	—	—	—	—	—
Inner	.819	.013	.457	.056	.127	.009	.155	.011	.140	.053	.112	.028	.224	.008	.149	.015	—	—	—	—	—	—
Gonozooids	.657	.047	.524	.017	.078	.018	.198	.022	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Avicularian zooids	.846	.032	.622	.032	.133	.019	.127	.016	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Reptadeonella bipartita																						
Edge zooids	.496	.033	.337	.019	.060	.005	.096	.010	.082	.026	.038	.009	.148	.011	.168	.109	—	—	—	—	—	—
Inner zooids	.546	.036	.320	.024	.060	.008	.098	.008	.104	.024	.041	.006	.157		.100	.029	—	—	—	—	—	—

Trematooecia aviculifera																						
Edge zooids	.815	.092	.586	.046	.174	.008	.207	.018	.100	.038	.040	.007	.285	.020	.173	.024						
Inner zooids + ovicell	.837	.069	.677	.084	.193	.021	.222	.015	.147	.018	.054	.013	.333	.060	.202	.052	.395	.044	.477	.070	—	
Ovicell memb. window																	.209	.027	.207	.028	—	
Steginoporella sp. nov.																						
A-Zooids:																						
Edge	1.080	.048	.688	.053	.486	.036	.605	.055	.022	.005	.008	.001	.584	.065	.528	.041	—	—	—	—	—	—
Inner	1.138	.039	.747	.022	.529	.016	.670	.023	.025	.008	.015	.005	.524	.061	.479	.058	—	—	—	—	—	—
B-Zooids																						
Edge	1.890	—	.972	—	.810	—	.900	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Inner	1.933	.149	.936	.065	.954	.074	.911	.084	.032	.006	.013	—	.514	—	.469	—	—	—	—	—	—	—
Steginoporella magnilabris																						
A-Zooids																						
Edge	.871	.086	.533	.001	.414	.013	.468	.025	.022	.005	.013	.006	.395	.028	.346	.057	—	—	—	—	—	—
Inner	.817	.076	.580	.082	.367	.037	.450	.055	.027	.013	.013	.005	.388	.030	.264	.020	—	—	—	—	—	—
B-Zooids																						
Edge	1.184	.047	.644	.060	.583	.027	.594	.051	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Inner	1.116	.055	.587	.050	.500	.030	.536	.069	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Stylopoma spongites																						
Edge zooids	.610	.076	.382	.052	.122	.017	.120	.014	.055	.009	.021	.002	.164	.055	.106	.052	—	—	—	—	—	—
Inner zooid + ovicell	.602	.020	.422	.023	.146	.009	.120	.010	.073	.009	.025	.060	.211	.012	.147	.025	.586	.012	.497	.020	.182	—

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PHYSIOLOGICAL AND MORPHOLOGICAL STATE OF THE SYMBIOTIC BACTERIA FROM LIGHT ORGANS OF PONYFISH

PAUL V. DUNLAP*

Department of Biology, University of California at Los Angeles, Los Angeles, California 90024

ABSTRACT

Symbiotic, bioluminescent bacteria (*Photobacterium leiognathi*) within and directly removed from the light organs of freshly sacrificed Philippine and Japanese ponyfish (family Leiognathidae) were analyzed for light production, oxygen uptake, morphology, and density. Luminescence averaged 2.4×10^4 quanta $\cdot s^{-1} \cdot cell^{-1}$ for bacteria from 24 fish (6 species in 3 genera), more than 10 times the maximum luminescences of *P. leiognathi* grown in culture. Light production (depending on the *in vivo* quantum yield for luminescence, 0.1 to 1.0) accounted for 1.7 to 17% of the total oxygen utilized by bacteria from the light organ, substantially more than found for *P. leiognathi* in culture. Bacteria from the light organ were non-motile, non-flagellated coccobacilloid to short rod-shaped cells (1.6×3.2 μm), whereas in culture they showed motility and polar flagellation. *In situ* doubling time for the population of light organ bacteria was estimated to be approximately one day, or 20 to 30 times slower than in culture. Within the tubules of the light organ, the bacteria were solidly packed inside elongate, thinly-walled saccules, with one to 20 saccules tightly filling each light organ tubule. The saccules held the bacteria at a density (calculated from bacterial cell and saccule volumes) of approximately 1×10^{11} cells $\cdot ml^{-1}$, which is a density roughly 15 times greater than estimated from total light organ volume. These findings lead to a maximal-luminescence, minimal-growth bacterial model of this symbiosis.

INTRODUCTION

Members of more than 30 families of marine fish utilize luminous bacteria for light production. The fish house their species-specific symbiotic bacteria in highly structured, specialized light organs and utilize the bacterial light in a variety of ways (Herring and Morin, 1978; Nealson and Hastings, 1979; Hastings and Nealson, 1981). For one of these associations, the light organ symbiosis between ponyfish and *Photobacterium leiognathi,* the host fish are readily accessible and much behavioral and anatomical information on them has recently been obtained (McFall-Ngai, 1983a, b; McFall-Ngai and Dunlap, 1983, 1984; Dunlap and McFall-Ngai, 1984). These studies provide a background for examination of ponyfish light organ bacteria.

Ponyfish (family Leiognathidae) are a group of small, schooling fish abundant in coastal waters of the Indo-West Pacific (for references, see Pauly and Wade-Pauly, 1981). Ponyfish emit several different bioluminescent displays that may function in feeding, defense, and reproduction (Hastings, 1971; Herring and Morin, 1978; McFall-Ngai and Dunlap, 1983). The versatility in types of luminescent displays results from the control over light emission provided by muscular shutters and chromatophores

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Abbreviations: BASW, buffered artificial sea water; PHB, poly-β-hydroxybutyrate; SWC, sea water complete medium.

* Current address: Department of Microbiology, New York State College of Agriculture and Life Sciences, Cornell University, Ithaca, New York 14853.

that cover the light organ, from the internal location of the light organ, and from several unique anatomical adaptations of the gas bladder, body musculature, and skin of the fish (Haneda, 1940; McFall-Ngai, 1983a, b; McFall-Ngai and Dunlap, 1983, 1984; Dunlap and McFall-Ngai, 1984). The internal, poorly vascularized ponyfish light organ overlies the esophagus just anterior to the stomach and interfaces with the oxygen-rich gas bladder across a thin, window-like membrane that is permeable to oxygen (McFall-Ngai, 1983a, b). Within the light organ, the dense, pure culture of *P. leiognathi* (Boisvert *et al.*, 1967; Hastings and Mitchell, 1971; Reichelt *et al.*, 1977; Jayabalan *et al.*, 1978) is held in numerous fine-diameter tubules that coalesce and empty into the esophagus via small ducts, thus allowing the bacteria to pass into the gut of the host (Harms, 1928; Haneda, 1940, 1950; Ahrens, 1965; Bassot, 1975). A facultative symbiont, *P. leiognathi* survives free of the host's light organ in various other marine habitats (Hastings, and Nealson, 1981).

The behavioral value of luminescence, and the anatomical and physiological commitment of the fish to maintaining light organ bacteria, indicate the importance of bacterial light production to the fish. Consequently, given the fish's secondary control of the intensity and type of light emission by muscular shutters and chromatophores over the light organ, maximal luminescence would appear to be adaptive in this association. In this regard, alternative rapid-growth and nutrient-limitation models for the ponyfish symbiosis have been proposed that are consistent with high levels of luminescence (Nealson, 1979). However, essentially no information is available on the intensity of bacterial luminescence *in situ* or on the control of luminescence by the fish at the primary (physiological) level.

The present study addresses questions of the physiological and morphological state of symbiotic bacteria in the ponyfish light organ. Measurements of bacterial light production and oxygen uptake, as physiological parameters of bacterial activity in the functioning symbiosis, are reported. The morphology, spatial distribution, and population density of light organ bacteria from several different ponyfish are described and interpreted in light of a newly discovered structural feature of this symbiosis, the light organ saccule. The results indicate that ponyfish bacteria grow slowly but produce consistently and exceptionally bright luminescence in the light organ, possibly in response to both anatomical and physiological controls exerted by the host fish. The results also provide a basis for *in vitro* studies of bacterial luminescence and growth that may lead to a better understanding of the physiology of this symbiosis.

Materials and Methods

Collection and handling of fish

The fish examined in this study were identified from the descriptions by Munro (1964), Kühlmorgen-Hille (1974), James (1975), Abe (1976), Rau and Rau (1980), and Dunlap and McFall-Ngai (1984). Ponyfish captured live from Manila Bay, Luzon and Bais Bay, Negros Oriental, Philippine Islands (*Gazza achlamys, G. minuta, Leiognathus bindus, L. brevirostris, L. equulus, L. fasciatus, L. splendens,* and *Secutor insidiator*), and at Misaki, Honshu, Japan (*L. nuchalis*), were maintained in laboratory aquaria near the collection sites as described by McFall-Ngai and Dunlap (1983) or were transported live to laboratory facilities at the University of California, Los Angeles and maintained under similar conditions. For morphological examination of bacteria, light organs from freshly sacrificed specimens of these fish and from fresh specimens of *L. elongatus* and *L. rivulatus* from Suruga Bay and the Sagami Sea, Japan, were preserved in potassium-phosphate buffered 10% formalin, pH 7.0. Only healthy specimens of the fish, held less than one week (typically 1 to 2 days), were used for

physiological and morphological analysis of live light organ bacteria. Live fish were individually netted from the holding aquarium and were quickly sacrificed by severing the spinal cord. Light organs were aseptically dissected from the fish, and esophageal and light organ shutter tissues were dissected away. Dissection typically took less than three minutes from the time of sacrifice. Width (lateral dimension), height (dorsal to ventral dimension), and length (anterior to posterior dimension) of the light organ were multiplied to calculate its volume, a method that may slightly overestimate volume due to the inclusion of the small esophageal space and because the light organ is only very roughly cuboidal. Immediately after being measured, light organs used in analysis of bacterial luminescence, oxygen uptake, and density were homogenized thoroughly in a sterile 8 ml Ten Broeck tissue homogenizer with 2.4 ml of 40% buffered artificial sea water (40% BASW = 11.0 g NaCl, 0.4 g KCl, 0.5 g $CaCl_2 \cdot 2H_2O$, 0.8 g $MgSO_4 \cdot 7H_2O$ to one liter with distilled water, and with 25 m*M* Tris · HCl, pH 7.5).

Luminescence and oxygen uptake

Using a photomultiplier-photometer similar to that of Mitchell and Hastings (1971), light production by bacteria was measured on light organ homogenates (in 40% BASW) diluted 1:1 or 1:3 with 40% BASW to eliminate possible quenching of luminescence (1 ml total volume). Using 40% BASW, replicate luminescence intensity measurements of aliquots of light organ homogenates were the same within ±5% when taken in the first minute after homogenization of the light organ. Thereafter, and more rapidly with homogenization fluid of higher salt content, luminescence declined markedly regardless of the extent of aeration. Therefore, all measurements of luminescence reported here were taken within 15 s following homogenization of the light organ. Luminescence is expressed as quanta $\cdot s^{-1} \cdot cell^{-1}$. The photometer was calibrated with light standard #231, which emits 9.7×10^8 quanta $\cdot s^{-1}$ ($\pm 0.2 \times 10^8$) at 480 nm (prepared and calibrated by J. W. Hastings, Harvard University). In a preliminary report of this study (Dunlap, 1983), light standard 'J' was used, but was found to overestimate light levels approximately 10-fold compared to standard #231. Because of this discrepancy and the present lack of uniform light standards, the more conservative standard #231 was used in the present report. Standard #231, however, may under- or over-estimate absolute light levels by 2 to 3 fold (K. H. Nealson, Scripps Institute of Oceanography, pers. comm.).

Using a polarographic oxygen electrode apparatus (Rank Brothers, Cambridge, England) calibrated to air-saturated 40% BASW, oxygen uptake by bacteria was measured on aliquots of light organ homogenates. The rate of oxygen uptake was measured over a 5 to 10 min period, and the reaction chamber temperature was maintained at the corresponding fish's aquarium temperature (27°C to 31°C, depending on the experiment). For direct comparison with luminescence, which uses oxygen as a reaction substrate (for reaction see Hastings and Nealson, 1981), oxygen uptake is expressed as molecules $O_2 \cdot s^{-1} \cdot cell^{-1}$. In certain cases, by suspending the organ in 40% BASW in the oxygen electrode reaction chamber, oxygen uptake was determined for intact light organs. Subsequently, those light organs were homogenized and handled as described above, and luminescence measurements were made. Oxygen uptake rates for bacteria released from the light organ directly into 40% BASW and for bacteria in association with tissue dissected from the light organ were essentially identical, which indicates that light organ tissue did not contribute measureably to oxygen uptake.

Morphology and population density of symbiotic bacteria

Scanning electron microscopy (SEM) was performed on formalin-preserved light organs that had been fixed for four hours in 2% glutaraldehyde (0.05 *M* Na-cacodylate buffer, pH 7.0). After three washes with buffer, the organs were post-fixed in 2% osmium tetra-oxide (0.05 *M* Na-cacodylate buffer, pH 7.0), again washed three times with buffer, and then dried through an ethanol series (10% to 100% ethanol). The light organs were then critical-point dried by carbon dioxide exchange in a Tousimis Samdri PVT-3 critical point dryer and sputter-coated with gold-palladium (50:50) in a Technics Hummer 1 sputter coater. The material was examined with an Etec Autoscan scanning electron microscope operated at 10 kV.

Using a phase contrast Zeiss microscope calibrated with ocular and stage micrometers, both freshly dissected and preserved light organs were examined for bacterial cell shape and dimensions, the number of bacteria visibly in division and the dimensions of light organ saccules. Flagellar staining of bacteria removed from freshly dissected light organs and, for positive controls, of bacteria cultured overnight on sea water complete (SWC; Nealson, 1978) agar and broth were carried out using the Leifson method (Doetsch, 1981).

For starvation studies, specimens of *L. splendens* were held in 30-gallon sea water aquaria with aeration, but without food for three weeks. Beginning two days after capture, two fish were sacrificed every two days and their opened light organs were examined visually for luminescence in a well-darkened room. To confirm the presence of symbiotic bacteria, contents of the light organs were streaked on SWC agar and examined for luminescent bacterial growth the following day.

Counts of viable bacteria, to estimate the total number of bacteria present in a light organ and their density (bacteria $\cdot$ ml^{-1} of light organ volume), were made by spread-plating 0.1 ml of the appropriate serial dilutions of light organ homogenates on duplicate SWC agar plates. Platings were completed within 10 min of homogenization of the light organ. Colonies were counted after 12 to 16 hours of incubation at 28°C; more than 99.9% were luminescent and uniform in colony morphology (>1500 colonies examined per plating), although in many instances the intensity of luminescence ranged from dim to very bright for colonies on the same SWC agar plate. A few randomly picked colonies from platings of homogenates from several different light organs were retained for taxonomic identification; all were confirmed as *P. leiognathi* according to the methodology of Reichelt and Baumann (1973) and Baumann and Baumann (1981), as previously reported for ponyfish light organ bacteria (Reichelt *et al.*, 1977). Using a Petroff-Hauser counting chamber, total direct counts of bacteria from light organ homogenates were found to be equal to viable counts (n = 3), indicating that 100% of the light organ bacteria are viable. Stomachs and intestines of several fish specimens were also removed aseptically, homogenized, and plated as described above. Freshly voided fecal pellets were handled in a similar way.

Results

Luminescence and oxygen uptake by symbiotic bacteria

Ponyfish bacteria directly removed from the light organ were consistently very brightly luminescent. For bacteria from 24 fish (in three genera and six species), the mean light intensity was 2.4×10^4 quanta $\cdot$ s^{-1} $\cdot$ cell^{-1} ($\pm$S.D.: 1.2×10^4; range: 0.9×10^4 to 4.7×10^4). This value was at least 10 times greater than maximum levels of luminescence produced by ponyfish bacteria in culture ($<10^3$ quanta $\cdot$ s^{-1} $\cdot$ cell^{-1})

and by bacteria taken directly from the light organ of the apogonid fish, *Siphamia cephalotes* (2.3×10^3 quanta $\cdot s^{-1} \cdot cell^{-1}$; Fitzgerald, 1978), which have been tentatively identified also as *P. leiognathi* (Reichelt and Baumann, 1973; Fitzgerald, 1978). Similar levels of light production (10^4 to 10^5 quanta $\cdot s^{-1} \cdot cell^{-1}$) have been reported for various bacteria in culture (Nakamura and Matsuda, 1971; Hastings and Nealson, 1977; Karl and Nealson, 1980), but meaningful comparisons between studies must await the availability of uniform light standards.

For 10 of these 24 ponyfish, both the luminescence and oxygen uptake rates of the symbiotic bacteria were determined. Luminescence showed a positive correlation with oxygen uptake (correlation coefficient, $r = 0.84$), and the levels of luminescence and oxygen uptake appeared to relate to the species of fish housing the bacteria (Fig. 1). Using a range of 0.1 to 1.0 for the as yet unknown *in vivo* quantum yield of luminescence (for discussion see Hastings and Nealson, 1977; Karl and Nealson, 1980), luminescence accounted for 1.7 to 17% of the total oxygen taken up by the bacteria (Table I). However, oxygen uptake by bacteria within intact light organs was half that of bacteria in light organ homogenates (Table II), so the percentage of oxygen consumed due to luminescence might be as high as 3.4 to 34%. In contrast, when grown in culture to peak luminescence, *P. leiognathi* consumes substantially less oxygen in light production, 0.02 to 0.2% (manuscript in prep.). Thus, the activity of ponyfish light organ bacteria *in situ* is characterized both by brighter luminescence and by a higher proportion of oxygen consumed in light production compared to *P. leiognathi* in culture. Earlier reports of oxygen consumption in luminescence by maximally luminescent bacteria in culture give generally similar percentages (2 to 53%: Eymers and van Schouwenberg, 1937; Harvey, 1952; Watanabe *et al.*, 1975; Karl and Nealson, 1980).

Morphology and density of symbiotic bacteria in the light organ

The symbiotic bacteria reside at high density inside narrow-diameter light organ tubules (Fig. 2), of which there are 150 to 200 (Bassot, 1975). External diameters of

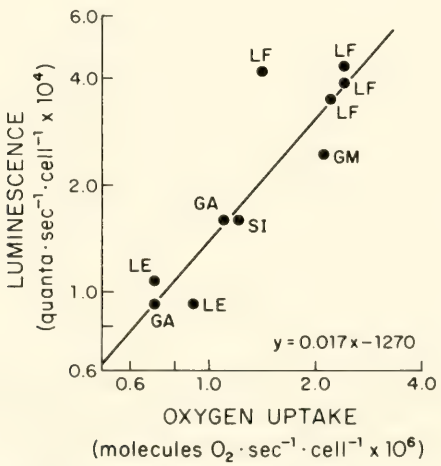

FIGURE 1. Plot of oxygen uptake *versus* light production for bacteria released from light organs of 10 ponyfishes. Fish species designations: GA, *Gazza achlamys;* GM, *G. minuta;* LE, *Leiognathus equulus;* LF, *L. fasciatus;* SI, *Secutor insidiator.*

TABLE I

Activity of symbiotic bacteria taken directly from ponyfish light organs

	Luminescence (quanta · s^{-1} · $cell^{-1}$) × 10^4	Oxygen uptake (O_2 · s^{-1} · $cell^{-1}$) × 10^6	O_2 consumed in luminescence (% of total)
$\bar{X}$[a]	2.5	1.5	1.7–17[b]
(±S.D.)	(1.4)	(0.7)	
range	0.9 to 4.4	0.7 to 2.4	

[a] n = 10 fish (see also Fig. 1).

[b] Lower and upper limits. *In vivo* quantum yield for luminescence is unknown but probably falls between 0.1 and 1.0 (see Hastings and Nealson, 1977; Karl and Nealson, 1980).

tubules in freshly dissected light organs ranged from 25 to 70 μm, a size similar to the 35 μm diameter reported by Bassot (1975) for tubules in light organs prepared for transmission electron microscopy (TEM). However, much variation in tubule wall thickness and tubule lumen diameter was noted for the different species of fish.

Bacteria taken directly from light organs of freshly sacrificed ponyfish and examined with phase contrast microscopy were, in general, uniformly shaped coccobacilloid to short rod-shaped cells averaging 1.6 × 3.2 μm. Among the fish examined, only the bacteria of *L. splendens* and *L. elongatus* varied much from the regular coccobacilloid shape. A high proportion of *L. splendens* bacteria (from light organs of seven juvenile specimens) were spherical, cube-shaped, pear-shaped, or blunt-ended, with distortion possibly due to tight cell packing during growth in the light organ tubules. Similar distortions of bacterial cell shape were noted by Bassot (1975). Bacteria of *L. elongatus,* which have only recently been brought into culture (Dunlap, 1984), were distinctly more elongate than those of other ponyfish (as also shown by Haneda and Tsuji, 1976). Bassot (1975) also noted the occasional presence of apparently lysed bacteria and 'ghost membranes' in the light organ of *L. equulus.* In the present study, such forms were seen only once, among otherwise normal-looking bacteria of one specimen of *L. fasciatus.* In this case, the 'ghost' bacteria represented approximately 60% of the bacteria from one tubule, whereas bacteria from other tubules of the light organ of this fish appeared normal.

As judged by phase contrast examination of wet mounts made from freshly dissected light organs (from 25 fish and several thousand bacteria examined per light organ), ponyfish bacteria lacked visible granules of poly-β-hydroxybutyrate (PHB) (also shown to be absent in TEM micrographs by Bassot, 1975; see Nealson, 1979) and in all

TABLE II

Rates of oxygen uptake of bacteria in intact and homogenized ponyfish light organs

Source of light organ	Intact organ (O_2 · s^{-1} · $cell^{-1}$ × 10^6)	Homogenized organ (O_2 · s^{-1} · $cell^{-1}$ × 10^6)	Intact:homogenized
Leiognathus equulus	0.5	0.9	0.55:1
L. equulus	0.3	0.7	0.43:1
Gazza achlamys	0.3	0.7	0.43:1
G. achlamys	0.5	1.0	0.50:1
			$\bar{X}$ 0.48:1

FIGURE 2. SEM of ponyfish (*Gazza minuta*) light organ tubules, which house the bacterial symbionts (*Photobacterium leiognathi*). Light organ saccules, apparently destroyed in preparation of the light organ for SEM, are not visible. Note the scarcity of capillaries (as noted by Bassot, 1975) and the density and uniformity of shape of the bacterial cells in the tubules. Size bar = 25 μm.

cases were non-motile. Using the Leifson method (Doetsch, 1981), staining for flagella gave negative results, whereas controls grown overnight in SWC broth and on SWC agar showed active motility and polar flagellation (as described for *P. leiognathi* in culture; Reichelt and Baumann, 1973). Motility of bacteria in light organ homogenates developed after 6 to 12 hours at 24°C.

Four to five percent of the 1200 bacteria examined from one fish (*G. minuta*) were visibly in division. Using a regression formula that relates frequency of dividing bacterial cells (FDC) to growth rate of a natural population, $\ln \mu = 0.299 \text{ FDC} - 4.961$ (Newell and Christian, 1981), the growth rate of the bacteria was estimated to be 0.03 h^{-1}. In culture, growth rates of 0.60 h^{-1} or higher are common for *P. leiognathi*. Thus, the light organ bacteria *in situ* double slightly less often than once

per day, at least 20 times slower than in culture. Using different methods, Haygood *et al.* (1984) found comparably slow *in situ* growth rates for *V. fischeri* from the light organs of pinecone fish (family Monocentridae).

Viable, brightly luminescent bacteria were retained in the light organs of all unfed specimens of *L. splendens* up to the point of death by starvation and cannibalism (3 weeks). With respect to the digestive tract, roughly equal volumes of the stomachs and intestines of several freshly sacrificed specimens of *L. nuchalis* held approximately equal numbers of luminous bacteria (2 to 3 $\times$ 10^5 cells). Additionally, fecal pellets released from various live, freshly captured ponyfish were brightly luminescent. 70 to 100% of the bacterial colonies that formed on SWC agar from homogenates of the stomach, intestines, and fecal pellets were luminescent and appeared identical in intensity and color of luminescence and in colony morphology to colonies formed by the light organ bacteria from the same fish. Although these observations require better quantification and taxonomic confirmation, they indicate that *P. leiognathi* may grow slowly in the light organ and, once released from the light organ, may pass through the gut and survive digestion in substantial numbers.

Estimates were made of the light organ volumes of 16 ponyfish and the total number and density of symbiotic bacteria in the organs (Table III). Although the fish specimens were of different species and sizes (which can influence light organ volume; McFall-Ngai and Dunlap, 1984), light organ volume was found to increase in parallel with the standard length of the fish ($r = 0.88$). The calculated total number of bacteria per light organ (Table III) increased as light organ volume increased ($r = 0.73$) and was comparable to values reported for ponyfish from New Guinea (Hastings and Mitchell, 1971) and from India (Jayabalan *et al.*, 1978). In addition, the density of bacteria (bacteria $\cdot$ ml^{-1}) in these light organs (Table III) was similar to that reported for other ponyfish (Hastings and Mitchell, 1971), for pinecone fish (Ruby and Nealson, 1976), for flashlight fish (family Anomalopidae) (Herring and Morin, 1978), and for *P. leiognathi* grown in SWC broth culture to stationary phase (4 to 5 $\times$ 10^9 cells $\cdot$ ml^{-1}). Using a mean bacterial cell volume of 6 to 7 μm^3 (6 to 7 $\times$ 10^{-9} μl), ponyfish bacteria of the present study were calculated to take up 4 to 5% of the volume of the light organ. This estimate may be low due to a possible overestimation of light organ volume (see Materials and Methods). Using a comparison between light organ wet weight and a packed-cell wet weight count of luminous bacteria of 5 $\times$ 10^{10} cells/g, Hastings and Mitchell (1971) estimated that ponyfish bacteria make up 10 to 25% of the mass of the light organ.

TABLE III

Quantification of bacteria in light organs of ponyfish[a]

	Standard length of fish (mm)	Light organ volume (ml)	Bacteria per light organ ($\times 10^8$)	Bacteria per ml in light organ ($\times 10^9$)	Bacteria per ml in saccules[b]
$\bar{X}$	79	0.036	2.5	6.4	1×10^{11}
(±S.D.)	(15)	(0.013)	(2.2)	(4.4)	
range	49–98	0.013–0.056	0.13–8.1	1.0–15.0	

[a] n = 16 fish: *Leiognathus equulus*, 3; *L. fasciatus*, 7; *L. splendens*, 1; *Gazza achlamys*, 3; *G. minuta*, 1; *Secutor insidiator*, 1. See text for details.

[b] Calculated on the basis of bacterial cell and saccule volume and with the assumption that interstitial space between bacteria accounts for 26 to 40% of saccule volume.

However, instead of being freely dispersed in light organ tubules as is suggested by their appearance in SEM (Fig. 2) and TEM micrographs (Bassot, 1975), the bacterial symbionts were found to be tightly packed within numerous long, thin, bag-like structures (Figs. 3, 4) that have not previously been described for ponyfish. Apparently similar, spherically shaped structures have been reported for light organs of the macrourid fish, *Physiculus japonicus, Coelorhyncous kishinouyei,* and *Malacocephalus laevis* (Kishitani, 1930; Yasaki and Haneda, 1935; Haneda, 1938), which harbor *P. phosphoreum* (Ruby and Morin, 1978; Herring and Morin, 1978). These structures were called 'sackchen' by Kishitani (1930), so the term 'light organ saccule' is used here. In gently dissected ponyfish light organs, all bacteria were contained within light organ saccules, and light organs of all fish examined (14 individuals from 6 species in 2 genera) exhibited these saccules. The saccules were transparent, apparently acellular (Fig. 3A), and were themselves tightly fitted into the tubules. Depending on tubule diameter, tubules contained from 1 to 20 saccules in tight bundles (Fig. 3B), which conformed to the dimensions of the internal diameter of the light organ tubules (20 μm for one saccule to 50 μm for a bundle of several saccules). Saccules appeared to run the entire length of the light organ tubule, at least 400 to 600 μm). Most saccules had a short extension at one end, which may be a secretion or attachment point (Fig. 4A). Due to the fragility of saccules, more precise quantification of their dimensions and number within light organ tubules was not possible. They were easily damaged during dissection and were distorted by pressure of the overlying cover slip during examination, which further revealed the tight packing of bacteria (Fig. 4B). Plate 3, figure 3 of Bassot (1975) shows what may be a saccule around the tightly massed bacteria within a tubule. Using comparisons between bacterial cell and saccule volumes, a saccule 20 μm in diameter by 600 μm in length was estimated to contain approximately 2×10^4 bacteria. Thus, to account for the total number of bacteria present in a light organ, approximately 1250 saccules of this size would be necessary. This number, which is 6 to 8 times larger than the number of light organ tubules cited by Bassot (1975), is consistent with the observation that many tubules hold more than one saccule.

Assuming that interstitial space between the tightly packed cells accounts for 26 to 40% of saccule volume, as for the packing of spheres (Sloane, 1984), the density of bacteria within a saccule was calculated to be approximately 1×10^{11} cells $\cdot$ ml^{-1} (Table III), a value 15 times higher than the density calculated on the basis of total light organ volume. This bacterial density within saccules is also much higher than the density estimated for bacteria of pinecone fish (Ruby and Nealson, 1976) and flashlight fish (Herring and Morin, 1978). However, no examination for saccules in the light organs of these or other bacterially bioluminescent fish (*e.g.,* apogonids, pempherids) except macrourids has been reported. Additionally, based on the estimated interstitial space within a saccule and the volume of the light organ taken up by bacteria (4 to 5%), the volume of fluid covering the bacteria within the saccules ('light organ fluid') of a typical light organ (0.036 ml, Table III) was calculated to be approximately 0.5 μl per light organ. The coccobacilloid shape of the bacteria may permit slightly closer packing than possible with spheres, so the volume of light organ fluid might be slightly less.

Discussion

This analysis of *P. leiognathi* within and directly removed from the ponyfish light organ provides insight into possible interactions between the host fish and symbiotic bacteria in the functional luminescent association. These interactions would appear

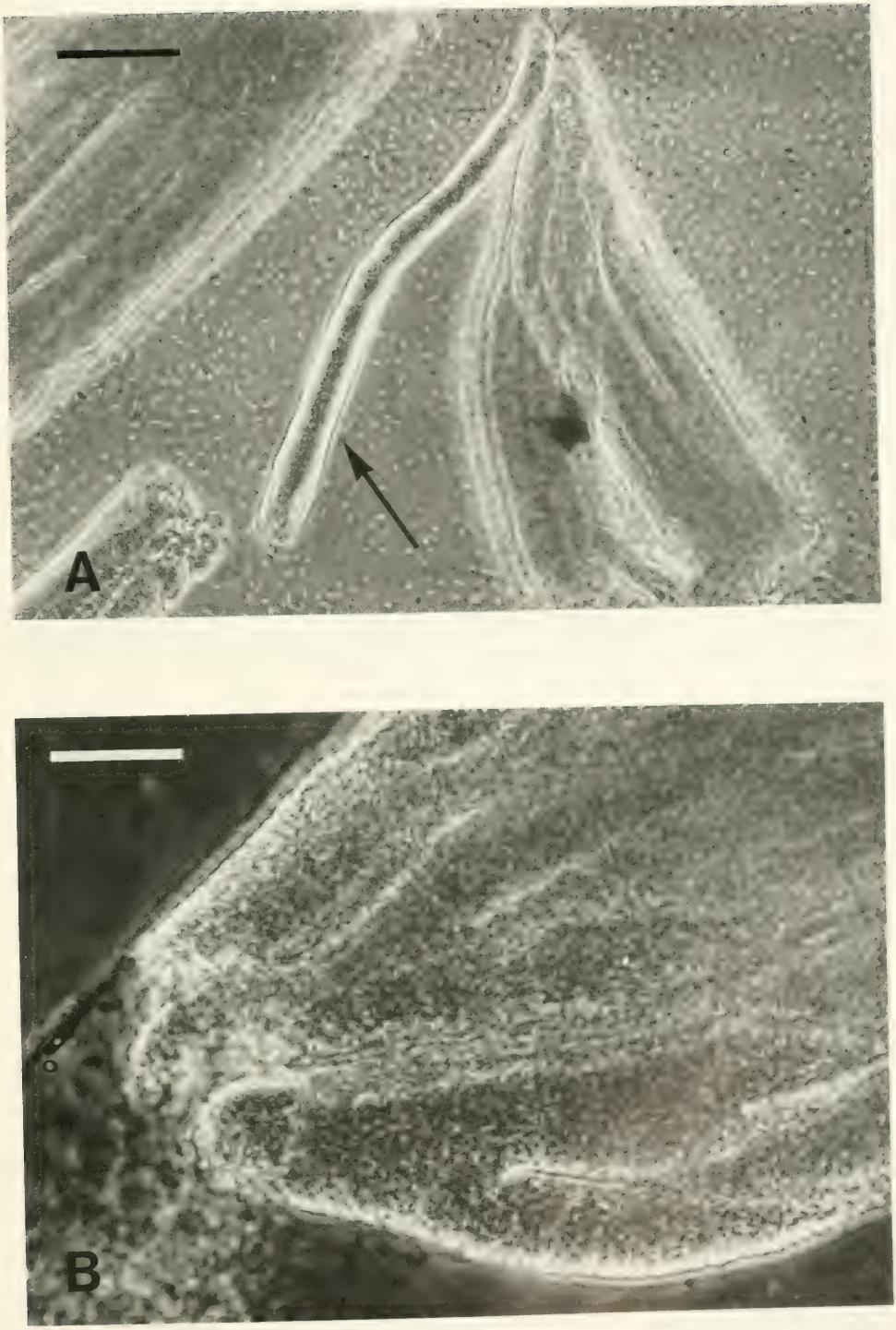

FIGURE 3. Ponyfish light organ saccules. (A) Bacterial symbionts are held inside elongate, thinly walled, transparent saccules (arrow), which are readily extruded from the light organ tubules (size bar = 75 μm); and (B) saccules, shown here extruded from the tubule, are present in bundles of up to 20 saccules per tubule (size bar = 20 μm). Note the solid masses of bacterial cells inside the saccules.

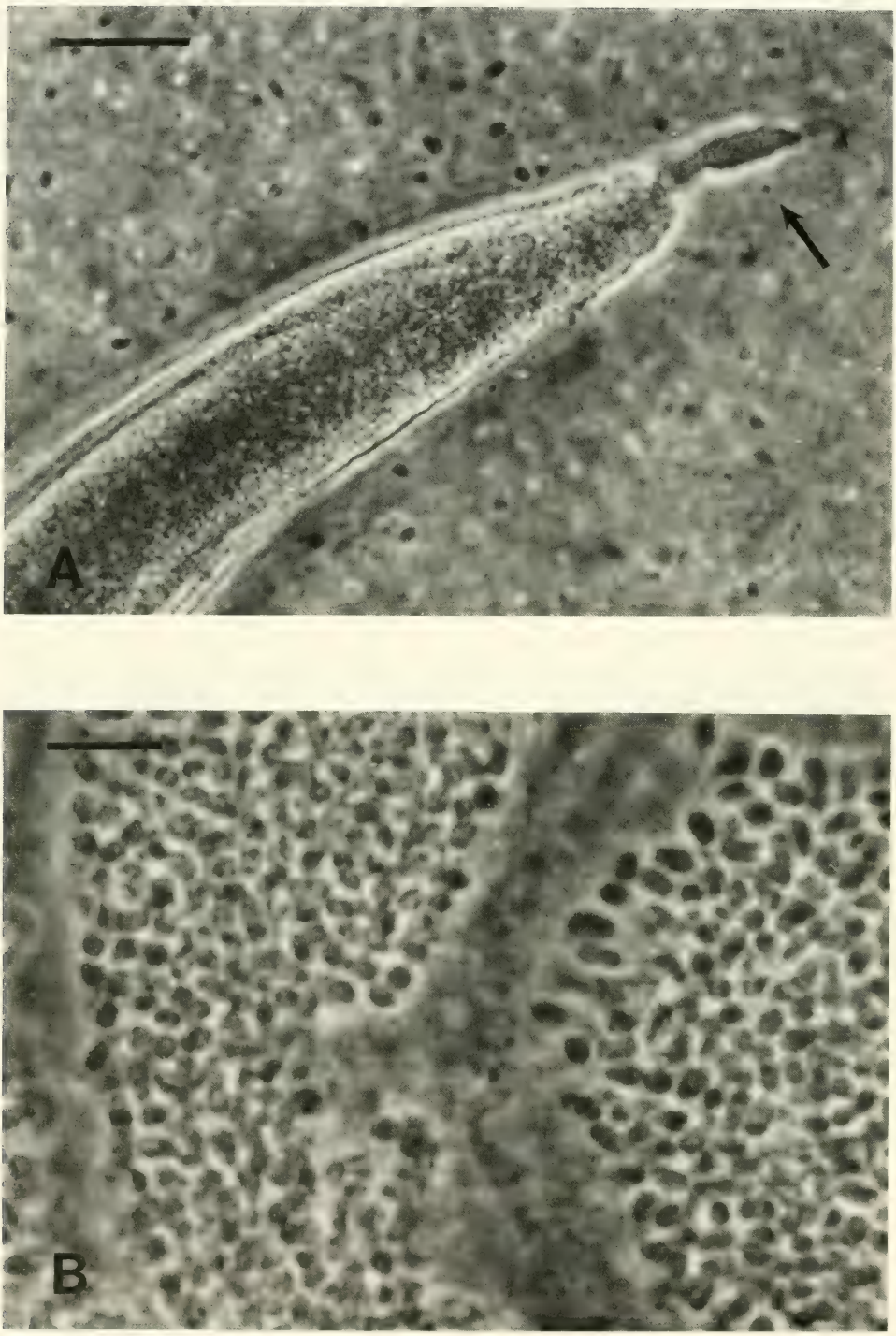

FIGURE 4. Ponyfish light organ saccules. (A) Each saccule bears a filament at one end (basal end?), possibly a site of attachment or secretion within the light organ tubule (size bar = 20 μm); and (B) the symbiotic bacteria occur in the saccule as tightly packed masses of coccobacilloid cells (size bar = 10 μm).

to derive from the controlling influences of micro-anatomical and as yet unknown physiological conditions of the light organ on bacterial luminescence and growth. Such control is reflected in the unusually bright luminescence and the higher percentage of oxygen consumed in luminescence by bacteria from the light organ, and in differences in their morphology and density within the light organ (Figs. 2–4), compared with in culture. The results of this study suggest a maximal-luminescence, minimal-growth model for bacterial activity in the ponyfish light organ and provide a basis for *in vitro* culture studies of the bacteria that may lead to a better understanding of the physiology of this symbiosis.

The fairly consistent and unusually high level of luminescence of bacteria from the light organ suggests that oxygen concentration in the light organ is sufficient for maximal induction and expression of the bacterial luminescent system. Ponyfish light organ bacteria appear to derive a substantial amount of their oxygen from the oxygen-rich gas bladder (McFall-Ngai, 1983b), which interfaces directly with the light organ across a thin oxygen-permeable membrane (McFall-Ngai, 1983a, b). In culture, oxygen concentration directly influences the luminescence of *P. leiognathi*, with higher levels of luciferase and brighter luminescence being produced when oxygen concentration is higher (Nealson and Hastings, 1977). Bacteria that produced more light took up more oxygen (Fig. 1), which suggests that a causal relationship might exist in the light organ between these two activities, with higher levels of available oxygen possibly leading to generally higher synthesis of luciferase and brighter cells. In this regard, the apparent trend for the bacteria of *L. fasciatus* and *G. minuta* to luminesce more brightly and take up more oxygen than the bacteria of the other fish (Fig. 1) could reflect species-based differences in oxygen concentration in the light organ.

At an oxygen concentration in the light organ sufficiently high for maximal luminescence, the symbiotic bacteria might exhibit rapid growth assuming other factors are not limiting; *P. leiognathi* grows rapidly in culture under such conditions (Nealson and Hastings, 1977). Rapid growth of the bacteria in the light organ could be a substantial energetic drain on the fish, one it might reduce by digesting the bacteria as they are released into the gut tract (Nealson, 1979). However, the apparently slow growth rate of bacteria in the light organ, their maintenance in the light organ during severe starvation of the fish, and their apparent ability to survive digestion are not consistent with this rapid-growth, digestion model. In contrast, these observations are consistent with an alternative, growth-limitation model in which the fish controls the growth of its light organ bacteria by restricting their supply of nutrients (Nealson, 1979) and consequently expends less energy in maintaining them.

The lower rate of oxygen uptake by bacteria in intact light organs compared to light organ homogenates (Table II) might indicate that the bacteria experience some oxygen limitation *in situ*. If so, the fish could be envisioned as controlling its bacteria by poising the oxygen concentration at a level that restricts growth but does not affect the expression or synthesis of luciferase. However, the available data indicating lower expression and synthesis of the luminescent system of *P. leiognathi* under low oxygen concentrations (Nealson and Hastings, 1977) are not consistent with this possibility. Alternatively, the removal of the light organ from the fish, and consequently from its normal oxygen delivery systems (gas bladder, circulatory system), may have created a surface-to-volume diffusion problem that accounts for this difference.

A newly discovered aspect of the micro-anatomy of the ponyfish light organ, the light organ saccule (Figs. 3, 4), may play a direct role in controlling luminescence by maximizing the density of bacteria. Light production by many other luminous bacteria has been demonstrated to be under the control of a density-dependent autoinduction system (Eberhard, 1972; Nealson, 1977; Ulitzur and Hastings, 1979; Rosson and

Nealson, 1981) and preliminary evidence is available for such a system in *P. leiognathi* isolated from ponyfish light organs (Dunlap, 1984). At the bacterial density found in light organ saccules, autoinducer concentration would presumably also be exceptionally high, thus maintaining the luminescent system of the bacteria in a maximally induced state. This form of control, coupled with an adequate level of oxygen for full induction and expression of the luminescent system, may partially or fully account for the observed high level of luminescence produced by the bacteria.

A second possible effect of light organ saccules may be to limit the flux of nutrients into the saccule lumen both by forming a diffusion barrier between the bacteria and host tissue and by maintaining a high bacterial density, which would keep the availability of nutrients low. That the bacteria of *L. splendens,* and of other ponyfishes (Bassot, 1975), are distorted in shape, due apparently to tight cell packing in the light organ during growth, emphasizes that the density of bacteria in the light organ is extremely high and distinguishes light organ growth conditions from those in culture on solid or liquid media. Saccules might also function to restrain the bacteria and allow nutrients to diffuse freely between them without permitting the bacteria to escape readily from the light organ tubules. The intense crowding of bacteria in the saccules may also account for the lack of motility, flagella, and poly-β-hydroxybutyrate (PHB) through density or nutrient-limitation effects. The shift to flagellated, motile cells some hours after escape from the light organ [and the development of PHB in culture (Reichelt and Baumann, 1973; Bassot, 1975; Nealson, 1979)] could involve release from these putative physical or nutritional constraints. Such possibilities are consistent with the nutrient-limitation model of this symbiosis proposed by Nealson (1979). The effects of nutrient limitation on light production, flagellation, and PHB deposition in these bacteria are, however, unknown, as are the type and fate of nutrients available.

Based on the measurements and observations of *P. leiognathi* presented here, the host fish is postulated to produce conditions under which the symbiotic bacteria luminesce maximally but grow at a minimal rate. The fish may evoke this response by presenting the bacteria with a growth-restrictive environment, possibly by limiting carbon directly (Nealson, 1979) or indirectly (high bacterial density in light organ saccules), or by iron-limitation (Haygood and Nealson, 1984). The apparently slow growth of the bacteria *in situ* and the sufficiently high oxygen concentration for maximal luminescence in the light organ implicate a form of growth restriction that acts at the level of the respiratory system. If control is manifested by such 'respiratory-restriction,' the bacteria might be responding adaptively by using the luminescent system as an alternative respiratory pathway to re-oxidize reduced coenzymes (as suggested for bacteria in culture by Hastings and Nealson, 1977, and Ulitzur *et al.,* 1981) and thus carry out maintenance metabolism and some growth. The very high level of luminescence produced by the bacteria and the high proportion of oxygen consumed in luminescence support this notion. Under these conditions, the fish would benefit doubly, first by minimizing the energetic cost of maintaining the bacteria and second by maximizing the physiological product (bioluminescence) that it obtains from them.

Although the nature of controls that could function to evoke a maximal-luminescence, minimal-growth response remain speculative at this time, the observations presented here should serve to stimulate comparisons between the physiological activity and morphological appearance of *P. leiognathi* in the ponyfish light organ and in culture. More specifically, the results of this study suggest that *in vitro* growth-restrictive culture conditions are likely to exist under which *P. leiognathi* will produce brighter luminescence than usual (*ca.* 10^4 quanta $\cdot s^{-1} \cdot cell^{-1}$). If these conditions exert their

control at the level of the respiratory system, oxygen would probably be taken up less rapidly, but proportionally more of it might be utilized for luminescence. As a consequence, *P. leiognathi* grown under such conditions might have reduced levels of functional cytochrome proteins, but would possibly be very rich in luciferase. Additionally, PHB and flagella might not be formed. Elucidation of the putative culture conditions evoking such responses from *P. leiognathi* may provide valuable insight into the nature of the controls used by the fish in this light organ symbiosis.

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LITERATURE CITED

ABE, T. 1976. *Keys to the Japanese fishes,* 6th ed. Hokuryukan Co., Tokyo. 374 pp.

AHRENS, G. 1965. Untersuchungen am Leuchtorgan von *Leiognathus klunzingeri* (Steindachner). *Z. Wiss. Zool.* **173:** 90–113.

BASSOT, J-M. 1975. Les organes lumineux à bactéries symbiotiques de quelques Téléostéens leiognathides. *Arch. Zool. Exp. Gen.* **116:** 359–373.

BAUMANN, P., AND L. BAUMANN. 1981. The marine gram-negative eubacteria. Pp. 1302–1331 in *The Prokaryotes,* M. P. Starr, H. Stolp, H. G. Truper, A. Balows, and H. G. Schlegel, eds. Springer-Verlag, New York.

BOISVERT, H., R. CHATELAIN, AND J-M. BASSOT. 1967. Étude d'un *Photobacterium* isolé de l'organe lumineux de poissons Leiognathidae. *Inst. Pasteur Paris* **112:** 520–524.

DOETSCH, R. N. 1981. Determinative methods of light microscopy. Pp. 21–33 in *Manual of Methods for General Microbiology,* P. Gerhardt, R. G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Krieg, and G. B. Philipps, eds. American Society for Microbiology, Washington, DC.

DUNLAP, P. V. 1983. Luminescence, respiration and density of *Photobacterium leiognathi* in light organs of leiognathid fishes: evidence for oxygen competition P. 160 in *Abstr. 83rd Ann. Meet. Am. Soc. Microbiol.,* New Orleans.

DUNLAP, P. V. 1984. The ecology and physiology of the light organ symbiosis between *Photobacterium leiognathi* and ponyfishes. Ph. D. dissertation, University of California, Los Angeles, California.

DUNLAP, P. V., AND M. J. MCFALL-NGAI. 1984. *Leiognathus elongatus* (Perciformes: Leiognathidae): two distinct species based on external morphological and light organ characters. *Copeia* **1984** (4): 884–892.

EBERHARD, A. 1972. Inhibition and activation of bacterial luciferase synthesis. *J. Bacteriol.* **109:** 1101–1105.

EYMERS, J. G., AND K. L. VAN SCHOUWENBURG. 1937. On the luminescence of bacteria II. Determination of the oxygen consumed in the light emitting process of *Photobacterium phosphoreum. Enzymologia* **1:** 328–340.

FITZGERALD, J. M. 1978. Studies on the taxonomy and bioluminescence of some luminous, marine bacteria. Ph.D. dissertation, Monash University, Victoria, Australia.

HANEDA, Y. 1938. Über den Leuchtfisch, *Malacocephalus laevis* (Lowe). *Jpn. J. Med. Sci.* **5:** 355–366.

HANEDA, Y. 1940. On the luminescence of fishes belonging to the family Leiognathidae of the tropical Pacific. *Palao Trop. Biol. Stn. Stud.* **2:** 29–39.

HANEDA, Y. 1950. Luminous organs of fish that emit light indirectly. *Pacific Sci.* **4:** 214–277.

HANEDA, Y., AND F. I. TSUJI. 1976. The luminescent systems of pony fishes. *J. Morphol.* **150:** 539–552.

HARMS, J. W. 1928. Bau und Entwicklung eines eigenartigen Leuchtorgans bei *Equula* spec. *Z. Wiss. Zool.* **131:** 157–179.

HARVEY, E. N. 1952. *Bioluminescence.* Academic Press, New York. 649 pp.

HASTINGS, J. W. 1971. Light to hide by: ventral luminescence to camouflage the silhouette. *Science* **173:** 1016–1017.

HASTINGS, J. W., AND G. MITCHELL. 1971. Endosymbiotic bioluminescent bacteria from the light organ of pony fish. *Biol. Bull.* **141:** 261–268.

HASTINGS, J. W., AND K. H. NEALSON. 1977. Bacterial bioluminescence. *Ann. Rev. Microbiol.* **31:** 549–595.

HASTINGS, J. W., AND K. H. NEALSON. 1981. The symbiotic luminous bacteria. Pp. 1332–1345 in *The Prokaryotes,* M. P. Starr, H. Stolp, H. G. Truper, A. Balows, and H. G. Schlegel, eds. Springer-Verlag, New York.

HAYGOOD, M. G., AND K. H. NEALSON. 1984. Effects of iron on bacterial growth and bioluminescence: ecological implications. Pp. 56–61 in *Proc. 3rd Int. Symp. Microbial Ecol.,* (*Curr. Persp. Microbial Ecol.*), M. J. Klug and C. A. Reddy, eds. Am. Soc. Microbiol., Washington, DC.

HAYGOOD, M. G., B. M. TEBO, AND K. H. NEALSON. 1984. Luminous bacteria of a monocentrid fish (*Monocentris japonicus*) and two anomalopid fishes (*Photoblepharon palpebratus* and *Kryptophaneron alfredi*): population sizes and growth within the light organs, and rates of release into the seawater. *Mar. Biol.* **75:** 249–254.

HERRING, P. J., AND J. G. MORIN. 1978. Bioluminescence in fishes. Pp. 273–329 in *Bioluminescence in Action,* P. J. Herring, ed. Academic Press, London.

JAMES, P. S. B. R. 1975. A systematic review of the fishes of the family Leiognathidae. *J. Mar. Biol. Assoc. India* **17:** 138–172.

JAYABALAN, Y., K. DHEVENDARAN, AND K. RAMAMOORTHI. 1978. Occurrence of symbiotic bioluminescent bacteria in Indian leiognathids. *Curr. Sci.* **47:** 648–649.

KARL, D. M., AND K. H. NEALSON. 1980. Regulation of cellular metabolism during synthesis and expression of the luminous system in *Beneckea* and *Photobacterium. J. Gen. Microbiol.* **117:** 357–368.

KISHITANI, T. 1930. Studien uber die leuchtsymbiose in *Physiculus japonicus* Hilgendorf, mit der Beilage der zwei neuen Arten der Leuchtbakterien. *Sci. Rep. Tohoku Univ. Sect. 4,* **5:** 801–823.

KÜHLMORGEN-HILLE, G. 1974. Leiognathidae. In *FAO Species Identification Sheets for Fishery Purposes, Eastern Indian Ocean (Fishing Area 57) and Western Central Pacific (Fishing Area 71), Vol. 2,* W. Fischer and P. J. P. Whitehead, eds. Food and Agriculture Organization of the United Nations, Rome.

MCFALL-NGAI, M. J. 1983a. Adaptations for reflection of bioluminescent light in the gas bladder of *Leiognathus equulus* (Perciformes: Leiognathidae). *J. Exp. Zool.* **227:** 23–33.

MCFALL-NGAI, M. J. 1983b. The gas bladder as a central component of the leiognathid bacterial light organ symbiosis. *Am. Zool.* **23:** 907.

MCFALL-NGAI, M. J., AND P. V. DUNLAP. 1983. Three new modes of luminescence in the leiognathid fish *Gazza minuta* (Perciformes: Leiognathidae): discrete projected luminescence, ventral body flash and buccal luminescence. *Mar. Biol.* **73:** 227–237.

MCFALL-NGAI, M. J., AND P. V. DUNLAP. 1984. External and internal sexual dimorphism in leiognathid fishes: morphological evidence of sex-specific bioluminescent signalling. *J. Morphol.* **182:** (in press).

MITCHELL, G. W., AND J. W. HASTINGS. 1971. A stable, inexpensive, solid-state photomultiplier photometer. *Anal. Biochem.* **39:** 243–250.

MUNRO, I. S. R. 1964. Additions to the fish fauna of New Guinea. *Papua New Guinea Argic. J.* **16:** 141–168.

NAKAMURA, T., AND K. MATSUDA. 1971. Studies on luciferase from *Photobacterium phosphoreum.* I. Purification and physico chemical properties. *J. Biochem.* **70:** 35–44.

NEALSON, K. H. 1977. Autoinduction of bacterial luciferase. Occurrence, mechanism and significance. *Arch. Microbiol.* **112:** 73–79.

NEALSON, K. H. 1978. Isolation, identification and manipulation of luminous bacteria. *Meth. Enzymol.* **57:** 153–166.

NEALSON, K. H. 1979. Alternative strategies of symbiosis of marine luminous fishes harboring light-emitting bacteria. *Trends Biochem. Sci.* **4:** 105–110.

NEALSON, K. H., AND J. W. HASTINGS. 1977. Low oxygen is optimal for luciferase synthesis in some bacteria. Ecological implications. *Arch. Microbiol.* **112:** 9–16.

NEALSON, K. H., AND J. W. HASTINGS. 1979. Bacterial bioluminescence: its control and ecological significance. *Microbiol. Rev.* **43:** 496–518.

NEWELL, S. Y., AND R. R. CHRISTIAN. 1981. Frequency of dividing cells as an estimator of bacterial productivity. *Appl. Environ. Microbiol.* **42:** 23–31.

PAULY, D., AND S. WADE-PAULY. 1981. An annotated bibliography of slipmouths (Pisces: Leiognathidae). *ICLARM Bibliographies, 2.* International Center for Living Aquatic Resources Management, Manilla, Philippine Islands.

RAU, N., AND A. RAU. 1980. *Commercial Marine Fishes of the Central Philippines (Bony Fishes).* German Agency for Tech. Coop., Eschborn.

REICHELT, J. L., AND P. BAUMANN. 1973. Taxonomy of the marine, luminous bacteria. *Arch. Mikrobiol.* **94:** 283–330.

REICHELT, J. L., K. NEALSON, AND J. W. HASTINGS. 1977. The specificity of symbiosis: pony fish and luminescent bacteria. *Arch. Microbiol.* **112:** 157–161.

ROSSON, R. A., AND K. H. NEALSON. 1981. Autoinduction of bacterial bioluminescence in a carbon limited chemostat. *Arch. Microbiol.* **129:** 299–304.

RUBY, E. G., AND J. G. MORIN. 1978. Specificity of symbiosis between deep-sea fish and psychrotrophic luminous bacteria. *Deep-Sea Res.* **25:** 161–171.

RUBY, E. G., AND K. H. NEALSON. 1976. Symbiotic association of *Photobacterium fischeri* with the marine luminous fish *Monocentris japonicus:* a model of symbiosis based on bacterial studies. *Biol. Bull.* **151:** 574–586.

SLOANE, N. J. A. 1984. The packing of spheres. *Sci. Am.* **250:** 116–125.

ULITZUR, S., AND J. W. HASTINGS. 1979. Autoinduction in a luminous bacterium: a confirmation of the hypothesis. *Curr. Microbiol.* **2:** 345–348.

ULITZUR, S., A. REINHERTZ, AND J. W. HASTINGS. 1981. Factors affecting the cellular expression of bacterial luciferase. *Arch. Microbiol.* **129:** 67–71.

WATANABE, H., N. MIMURA, A. TAKIMOTO, AND T. NAKAMURA. 1975. Luminescence and respiratory activity of *Photobacterium phosphoreum.* Competition for cellular reducing power. *J. Biochem.* **77:** 1147–1155.

YASAKI, Y., AND Y. HANEDA. 1935. On the luminescence of the deep-sea fishes, family Macrouridae. *Oyo Dobutsu Zasshi Tokyo* **7:** 165–177.

Reference: *Biol. Bull.* **167**: 426–436. (October, 1984)

LABORATORY STUDIES OF FEEDING AND MATING IN SPECIES OF *CARCINONEMERTES* (NEMERTEA: HOPLONEMERTEA)

PAMELA ROE

Department of Biological Sciences, California State College Stanislaus, Turlock, California 95380 and University of California, Bodega Marine Laboratory, Bodega Bay, California 94923

ABSTRACT

Details of the suctorial feeding behavior of some species of *Carcinonemertes* on crab eggs are described. Under laboratory conditions juvenile and male worms ate an average of 0.6–0.7 crab egg/day. Females ate 2–3 eggs/day. Laboratory feeding rates of worms from different hosts were similar when worms were fed eggs of the same host. No worms ate lobster eggs. Worms grown under laboratory conditions tended to be smaller than similar-aged worms found on hosts. Female worms fed natural host eggs or eggs of unnatural hosts started laying egg strings in a variable number of days. More variability in timing of egg production was observed within a worm species than between species when fed on the same host's eggs. Mating behavior is described for the first time for a species of *Carcinonemertes.*

INTRODUCTION

Along the West Coast of the United States many brachyuran crabs in several families harbor nemerteans of the genus *Carcinonemertes.* These include the grapsids *Hemigrapsus oregonensis, H. nudus,* and *Pachygrapsus crassipes* (Kuris, 1978; Roe, 1979); the cancrids *Cancer magister* (Wickham, 1978), *C. antennarius, C. anthonyi, C. productus* (Kuris, 1978), *C. gracilis* (Stricker and Reed, 1981), *C. jordani* (Roe, pers. obs.); the majid *Pugettia producta* (Coe, 1902; Roe, pers. obs.); and the leucosiid *Randallia ornata* (Roe, pers. obs.). *Carcinonemertes errans* is apparently host specific, being restricted to *Cancer magister* (Wickham, 1978). *Carcinonemertes epialti* was named by Coe (1902) for worms found on *Pugettia producta.* Worms on hosts other than *Cancer magister* are presently considered to be *C. epialti,* although slight differences exist in worms from different hosts (Wickham, 1978). The actual number of species of U. S. west coast carcinonemerteans and degree of host specificity are unknown, especially with respect to the worms called *C. epialti.*

During the crab brooding season, worms on ovigerous females feed, grow to maturity, and lay their own egg strings among the crab eggs (Humes, 1942; Kuris, 1978; Roe 1979; Wickham, 1980). The time required for worms to mature and reproduce is different for crabs with different brooding times, with worm larvae and crab zoeae tending to hatch at the same time. For example, worms on *Hemigrapsus oregonensis* with a brooding period of 44 days in central California, take about 44 days to feed and reproduce and for larvae to hatch (Kuris, 1978; Roe, 1979), whereas in *Cancer magister,* whose brooding season is about 85–90 days in central California, the worm's growth and reproductive period also takes about 85–90 days (Wickham, 1980). During their trophic period worms feed on developing crab embryos and the yolk within the crab eggs.

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Worms on male crabs are thought to be only juveniles since, at least with *Cancer magister,* most worms on male crabs transfer to female crabs at the time of crab mating, whereas none have been observed to transfer from female to male crabs (D. E. Wickham *et al.,* Univ. Calif. Bodega Marine Laboratory, in prep.). Worms on male crabs remain in the juvenile state, apparently being maintained by uptake of dissolved organic materials leaked through arthrodial membrane areas of host crabs (Roe *et al.,* 1981; Crowe *et al.,* 1982). Worms on non-brooding female crabs appear to be juveniles as well, although just after a female releases zoeae, her worms are post reproductive, and shrink. The life span of the worms is still unknown.

Members of all the species of *Carcinonemertes* used in this study exhibit sexual dimorphism. Females become larger than males and have a bluntly pointed posterior end. In addition, the gut diverticula appear more pronounced in females. Males are smaller, appear to have fewer and less regularly arranged gut diverticula, and the posterior end is truncate to concave. At maturity gametes also show through the body wall; the previously mentioned features appear before worms are fully mature.

Carcinonemertes errans exists in epidemic numbers on the commercially important dungeness crab, *Cancer magister,* in central California, Oregon, and Washington (Wickham, 1979), and because the worm eats crab eggs and exists in such large numbers, it has been implicated in the collapse of the central California dungeness crab fishery (Wickham, 1979). Efforts are being undertaken to understand the relationship between worms, host, and fishery and to control worms on *Cancer magister* (Kuris, 1981–1982). Mortality of crab eggs due to feeding by *C. errans* in nature has been estimated to be about 70 eggs/worm brooding season (Wickham, 1979), and Hamilton (1984) found similar feeding rates by *C. errans* under laboratory conditions.

This study was designed to (1) describe feeding behavior, (2) determine the basic feeding rates of worms from *Hemigrapsus oregonensis* on *H. oregonensis* eggs, (3) determine host egg specificity in feeding by *Carcinonemertes errans* and by worms on other available hosts, and (4) compare feeding between worms from different hosts. Results should provide information on some aspects of host specificity and on complexity of possible control measures for *C. errans.*

Materials and Methods

Hemigrapsus oregonensis was picked from under intertidal rocks at Bodega Harbor and Elkhorn Slough; *Cancer magister,* two males of *C. antennarius,* and one male *Randallia ornata* were collected in crab traps by commercial fishermen near Bodega Bay, and one brooding female of *Cancer jordani* was collected from a sea water intake screen at Bodega Marine Laboratory. Crabs were held in sea water tables at Bodega Marine Laboratory (12–14°C) or in aquaria in a constant temperature room at California State College Stanislaus (about 12°C).

Worms and crab eggs were removed from crabs by fine forceps and worms were held either individually or in groups with a small cluster of crab eggs. Most worms and eggs were kept in covered 5-cm disposable plastic petri dishes, although a few were held in one of the two different designs of flow-through worm holders designed by Hamilton (1984) and R. Okazaki (Bodega Marine Laboratory and U.C., Santa Barbara). The flow-through containers had the advantage of constant water exchange, but worms sometimes escaped. The petri dishes were large compared to the size of worms and clump of eggs each contained, and appeared to provide an adequate environment for the worms. Since the petri dish containers were easier to work with, they were used in most experiments. Sea water in petri dishes was changed occasionally. Containers with worms were kept on the sea water tables in Bodega Marine Laboratory

or in the constant temperature room at California State College Stanislaus. For quantitative feeding experiments the number of healthy crab eggs added to a container with a worm was recorded. After a time lapse of a few days, the number of partly or completely eaten eggs was recorded. Uneaten eggs or fresh eggs were then left in the container with the worm. When eggs are completely eaten only the egg membrane is left; when eggs are partly eaten a string of yolk is seen coming out of the egg, the contents inside are reduced, or there is a large clear space inside. Since the crab embryo is killed even if only partly eaten, and since, for most of the worm feeding period, nearly the entire contents of eggs are eaten, partly eaten eggs are considered eaten in this study. The terms egg or crab egg refers to fertilized, extruded eggs developing into embryos during the crab brooding period.

Many experiments were set up to see whether worms from one host would eat and develop if given eggs from another host. In these situations small clusters of eggs were added to containers with one or more worms as before, but were not counted. Time from feeding until the first worm egg string was produced was recorded for these experiments.

The following combinations of worms and eggs were made: worms from *Hemigrapsus oregonensis* with eggs from *H. oregonensis, Cancer jordani, Cancer magister,* and the lobster *Homarus americanus;* worms from *Cancer magister* with eggs from *C. jordani, H. oregonensis,* and *H. americanus;* one female and one male worm from *C. jordani* with eggs from *C. jordani* and *H. oregonensis;* worms from *Cancer antennarius* with *H. oregonensis* eggs; and one worm from *Randalli ornata* with *H. oregonensis* eggs. Since ovigerous females of *Hemigrapsus oregonensis* can be easily collected during any good low tide, their eggs were the most readily available and were the ones most frequently used in the experiments.

Some worms (25–50% of the total) in nearly all experiments would not feed, on natural or unnatural host eggs. For this reason, many worms were sometimes put in with a cluster of crab eggs for a few days before start of an experiment. Only worms that fed during the initial period were used in the experiment. Also, feeding rates were calculated only from worms that did eat.

Except for *C. errans,* worms were usually identified by their natural host rather than by species, since the actual number of species, and host specificity, is unknown for the group called *Carcinonemertes epialti.*

Results

Description of feeding behavior

The feeding behavior of species of *Carcinonemertes* when eating crab eggs is typical of suctorial feeding among nemerteans as described by McDermott (1976). A worm spreads its anterior end against a crab egg (see picture in Wickham, 1978), apparently makes a hole in the egg membrane with its stylet, then inserts its foregut into the egg (Wickham, 1979; Roe, pers. obs.). The proboscis of *Carcinonemertes* apparently does not secrete toxin: on one occasion, when a worm was feeding on old embryos, the embryo that was being fed upon was much more active than other embryos in the egg cluster.

The foregut is muscular; one can observe it to undergo peristalsis, to move around within the egg, and to be more and less everted often while it is within the egg. A rather strong suction is apparently created by the foregut: I have seen the pigment comprising the eye of a developing zoea being pulled out of the circular eye into an elongate stream of pigment particles flowing into the gut. In addition, yolk is sometimes pulled out of the egg beyond the egg membrane. The ciliated stomach is probably also important in drawing food into the worm.

Particles of yolk and more fluid parts of developing zoeae flow into the foregut and on into the worm. Since the yolk of *Hemigrapsus oregonensis* eggs is dark brown, it can easily be seen streaming into the gut through the thin, orange nemertean body wall. The gut fills from the posterior end, and the worm diameter increases as the gut fills. The gut can be filled to just posterior of the position of the middle chamber of the inverted proboscis, but worms sometimes quit feeding before the gut is completely filled.

Small worms tend to flatten their anterior end against an egg more than larger worms; the head of larger worms is often only barely wider than the body while the worm is feeding. The anterior end of the body can undergo peristalsis along with the foregut, or the worm can lie still during feeding. A worm can also lose contact several times with the prey egg before it is finished feeding. I have seen worms pull away from their food, then return to it immediately up to 7–8 times during a single feeding episode.

In feeding observations of three juveniles and one female it took approximately 30 minutes, from soon after feeding commenced until finished, when the worm pulled away from the egg, turned, and crawled away. Often a stream of yolk is left protruding from a partially eaten egg, especially if eggs are young (recently extruded, with much yolk material). Once, a second worm was observed to feed on protruding yolk made by another worm, before the first one was finished eating. Multiple use of crab eggs might be a partial explanation for the apparent depression in feeding rate with increased worm density observed by Wickham (1979).

Feeding by worms from Hemigrapsus oregonensis *on eggs of their natural host,* H. oregonensis

Data for laboratory feeding rates and duration of different life history stages of worms for worms taken from *Hemigrapsus oregonensis* are summarized in Table I. Individual worms (followed through development) that grew into males showed an increased feeding rate as they became mature; it took about 20 days before they had developed enough to be recognized as males. Males tended to eat in the first few days

TABLE I

Laboratory feeding rates of worms from Hemigrapsus oregonensis *on eggs of* H. oregonensis

Worm life stage	Average feeding rate (eggs/day ± 1 S.D.)	Number of worms in sample	Duration of life stage (days ± 1 S.D.)
JUVENILE			
overall	0.63 ± 0.26	16	—
juvenile to male	0.69 ± 0.22	5[1]	19.8 ± 4.49
juvenile to female	0.92 ± 0.25	3[2]	16.0 ± 3.50
MALE			
overall	0.74 ± 0.67	9	—
mature	1.3 ± 0.58	4[1]	14.3 ± 7.18
post-reproductive	0.2 ± 0.14	4[1]	19.3 ± 4.92
FEMALE			
overall	2.14 ± 0.71	10	—
mature	2.4 ± 1.0	3[2]	17.3 ± 1.53
post-reproductive	0.14 ± 0.10	4	11.8 ± 2.87

[1,2] Same individuals through time. Number of worms in sample is the same for Average feeding rate and Duration of life stage in each category.

after they could be recognized as males; after they reached assumed maturity, feeding dropped to almost nothing (see mature and post-reproductive males, Table I). Older males would move away from the eggs and become quiescent, usually at the edge of the container.

Juvenile worms that grew into females had a higher feeding rate as juveniles than those that developed into males (Table I), and it only took about 16 days from the start of experiments to recognize them as immature females. The trophic period of females was longer than that of males (Table I), and females consumed eggs at the greatest rate (and actual number) of any of the life history groups.

The feeding rate for all actively metabolizing, egg producing females (Table I, overall female category) included several worms that had matured on host crabs before experiments were started, so the duration of active feeding and egg laying was not determined for this group (Table I). However, as with males, after the reproductive period was over, females reduced their feeding rate considerably. In addition, of 8 post-reproductive females removed from crab hosts to see if they would feed on recently extruded crab eggs or crab eggs about to hatch, 7 did not feed for at least 16 days and 1 worm ate 11 young eggs in 25 days (=0.44 egg/day for that worm).

The first egg strings produced by the three females followed throughout their development (Table I) appeared about 31 days (Table II) after feeding started, and these females grown from juveniles in the laboratory laid an average of 5.6 (S.D. = 5.03) egg strings. Their reproductive output can be compared to an average of 6.4 (n = 5, S.D. = 1.140) egg strings laid by females taken from hosts after they had already developed into females. Experimental females tended to be smaller than females taken from hosts (average length of 3 females grown from juveniles = 4.67 mm, S.D. = 1.258; average length of mature females collected from host for feeding experiments = 6.5 mm; average length of 47 females of varying degree of maturity from host crab = 5.18 mm, S.D. = 1.59). Experimental males were also smaller than males from nature, (laboratory reared, average = 1.97 mm, S.D. = 0.43, n = 7; males from host crabs, average = 2.81 mm length, S.D. = 0.544, n = 43).

TABLE II

Average number of days ± 1 S.D. from start of experiment to production of first worm egg string, by worms fed eggs of various hosts

	Egg Species		
Worms from host crabs	*Hemigrapsus oregonensis*	*Cancer magister*	*Cancer jordani*
Hemigrapsus oregonensis	31 ± 1.00 (3)	16 or 29 (1)	14 (1)
Cancer magister	F-36.2 ± 9.43[1] (6)		
	W-42.0 ± 5.07[1] (8)		
	S-28.7 ± 3.86[1,2] (7)		
	O-36.4 ± 8.46 (21)	—	19 (1)
Cancer antennarius	23.2 ± 1.66[2] (11)	—	—
Randallia ornata	21 (1)	—	—

Numbers in parentheses are the numbers of worms in the samples; F = fall, W = winter, S = summer, O = overall.

[1] Difference between median number of days to reproduction fall/winter *versus* summer significant at 0.2 level, Mann-Whitney U Test.

[2] No significant difference between median number of days to reproduction in summer for worms from *C. magister versus C. antennarius*, 90% level, Mann-Whitney U Test.

From feeding rates and days of feeding, it was calculated that a typical worm developing into a female would consume approximately 57 eggs in 45 days and a typical worm growing into a male would eat 36 eggs in 53 days. Using only the subsample of two worms that actually grew from juveniles into adult, then post-reproductive males, the total egg consumption of males was 22 eggs in 34 days.

Feeding on eggs of Hemigrapsus oregonensis *by worms found on other hosts*

Individuals of *Carcinonemertes errans* were the most studied with respect to feeding rate, since *C. errans* is a known different species from worms on other hosts. Data for laboratory feeding rates and duration of various life history stages of *C. errans* eating eggs of *Hemigrapsus oregonensis* are summarized in Table III.

Those juveniles that developed into males had a somewhat lower feeding rate than the average juvenile (Table III). As they developed into adults, the feeding rate increased. It took longer for these animals to mature than it took worms from *H. oregonensis* (Table I), but they remained active feeding adults for about two weeks, as did the males from *H. oregonensis* (Table I). Post-reproductive worms quit eating (Table III). Juveniles that developed into females had a higher feeding rate than the other juveniles, and mature females had the highest feeding rate of all life history categories (Table III). Again, it took longer to recognize these animals as immature females than it did with worms from *H. oregonensis*, but they remained adults approximately the same amount of time (Tables I, III). In the four animals followed from juvenile through female stage, after they were recognizable as females but before they were sexually mature, the worms ate more than they did as juveniles but less than they did as mature females (see immature and mature female stages, Table III). Eleven females laid an average of 5.4 egg strings (S.D. = 0.75) and reproduction started about 36 days after feeding commenced (Table II).

Time to first reproduction varied considerably between test groups of *C. errans* (Table II). The difference between the median time before first reproduction in fall-winter *versus* summer was significantly different at the 0.2 level using the Mann-Whitney U Test. By contrast, the difference between the median time of first repro-

TABLE III

Laboratory feeding rates of Carcinonemertes errans *on eggs of* Hemigrapsus oregonensis

Worm life stage	Average feeding rate (eggs/day ± 1 S.D.)	Duration of life stage (days ± 1 S.D.)
JUVENILE		
overall	0.65 ± 0.30 (10)	—
juvenile to male	0.33 ± 0 (2)	24.8 ± 3.36 (28)
juvenile to female	0.84 ± 0.25 (5[1])	25.7 ± 4.24 (23)
MALE		
mature	0.66 ± 0.28 (4)	12 ± 2 (3)
post-reproductive	0 ± 0 (4)	4 (4)
FEMALE		
overall mature	3.2 ± 1.77 (6)	14.2 ± 5.42 (6)
immature	2.46 ± 1.83 (4[1])	8.3 ± 2.06 (4[1])
mature	3.5 ± 1.89 (4[1])	12.25 ± 2.06 (4[1])
post-reproductive	0.26 ± 0.23 (3)	7 ± 1.73 (3)

[1] Same individuals through time. Numbers in parentheses are the numbers of worms in the samples.

duction of summer-grown worms from *Cancer magister* and worms from *C. antennarius* was not significant (90% level, Mann-Whitney U Test, Table II, and section on feeding on alternate host eggs and reproduction, below).

After reproduction, females reduced feeding (Table III) before experiments were terminated. Experimental females grew to an average of 5.83 mm length (n = 6, S.D. = .97); experimental males to 2.6 mm (n = 4, S.D. = 0.35).

Using above figures the calculated egg consumption of a typical *C. errans* female through her life cycle is about 68 eggs in 48 days. Typical egg consumption of a male is about 16 eggs in 37 days.

Results of Mann-Whitney U Tests comparing median feeding rates of worms from *H. oregonensis* on natural host (*H. oregonensis*) eggs and of *Carcinonemertes errans* on *H. oregonensis* eggs showed no significant differences between the following pairs: juvenile, male, or mature female feeding rates (Table I, III; U statistic, two-tailed test, 90% significance level).

The one brooding female of *Cancer jordani* that was available harbored only a few worms, which were already mature at the time of collection. The one mature female worm tested ate an average of 3.4 *C. jordani* (natural host) eggs/day for five days. During this time she laid three egg strings. The same female was then presented with *H. oregonensis* eggs (and none from *C. jordani*); during the next seven days she ate an average of 3.1 *H. oregonensis* eggs/day and laid three more egg strings. After that she quit eating, even when presented with *C. jordani* eggs. Her feeding rate on *H. oregonensis* eggs was similar to that of worms from other hosts (Tables I, III), and her feeding rate was similar on her own host and on *H. oregonensis* eggs. Her size (7.2 mm) and six egg strings were also similar to other worms. In addition, the experiments with the *C. jordani* female indicate that imprinting on one species of host egg does not occur.

A male worm from the *C. jordani* ate 0.25 egg/day of *H. oregonensis* eggs for eight days. This animal was was thought to be a post-reproductive male since males appear to mature sooner than females, since the female from the same host was already mature, and since the crab's zoeae hatched within 18 days of the start of this experiment. Again, the feeding rate is similar to that of other post-reproductive male worms (Tables I, III).

One juvenile worm was found on a male *Randallia ornata.* It had a juvenile feeding rate of 0.79 *H. oregonensis* eggs per day, grew into a female, and laid one egg string within 21 days after feeding started.

Feeding on alternate host eggs and reproduction

In addition to eating eggs of their natural host, worms from *Hemigrapsus oregonensis* fed, grew, and reproduced on eggs of *Cancer magister* and *C. jordani.* About eight juvenile worms placed with a clump of *C. jordani* eggs became pale when feeding on the golden yolk of these eggs. Worms started making egg strings in 14 days. Of the worms in this experiment, two grew into males, 3 mm and 1.5 mm in length, and two grew into females, 3.5 mm and 5.5 mm in length.

About eight juveniles put with a cluster of *Cancer magister* eggs fed, grew, and reproduced on these eggs also. The eggs became fouled with fungus, bacteria, and ciliates soon after the experiment started, and worms did not appear to be feeding successfully on the fouled eggs. They remained small, although several of them could usually be found on the eggs rather than on the sides or bottom of the fingerbowl. The experiment was re-started with 4 of the original juveniles and fresh *C. magister* eggs after 13 days. Within six days after the fresh crab eggs were added the worms

were considerably larger and their guts were orange from the orange-colored egg yolk; within 10 days sexes could be determined (2 young females, 3.5 mm and over 2.5 mm, and 1 male, 1.5 mm). On the 10th day, between the time of removal of crab eggs and replenishment with fresh eggs (about 5 mins.), I realized the male was mating with the smaller female. Mating has never before been described for any species of *Carcinonemertes*. The worms became arranged with their posterior ends together. The male (especially the posterior part of the worm) underwent peristalsis to move backwards to touch the female. The front end of the male only moved if he lost contact with the female; in fact, his head anterior to his eyes seemed compressed and flattened (to gain traction?) on the dish bottom. The female was quiescent but did crawl forward (away from the male) a bit, then would back towards him again. The male would place his posterior end over a small area of the female. The posterior truncate-to-concave end of the male was quite active, and appeared to form a firmly attached muscular cup over each area contacted on the female. As the female backed up to the male, his posterior end appeared to service each gonopore along the female's side. Worm egg strings were produced 6 days later (16 days after the experiment was re-started with clean eggs or 29 days after the start of the experiment, Table II). Mature females were 3.5 and 5 mm long; the male remained 1.5 mm long.

Seven reproducing females from a female *H. oregonensis* were placed in a fingerbowl with recently extruded eggs of the American lobster, *Homarus americanus*. Although worms were sometimes found on these eggs, and although they continued to lay egg strings, none fed on the lobster eggs. Three individuals even seemed repelled by yolk from a broken egg, backing away then turning and crawling off after contact with the yolk.

In addition to *H. oregonensis* eggs, *Carcinonemertes errans* fed, grew, and reproduced on *C. jordani* eggs. Approximately 12 juveniles of *C. errans* ate and became pale from the light-golden yolk of *C. jordani* eggs. The first egg string was produced in 19 days (Table II). *C. errans* was not tested with eggs of its normal host, *C. magister*, because this had already been done (Hamilton, 1984).

Juveniles of *C. errans* placed with recently extruded eggs of *Homarus americanus* did not feed on the lobster eggs in seven days. Some juveniles were found among the eggs, but most were observed to be on the bottom or sides of the fingerbowl, away from the eggs.

Juveniles from *Cancer antennarius* placed with eggs of *Hemigrapsus oregonensis* produced an average of 5.3 (S.D. = 0.82, n = 6 females) egg strings in an average of 23.2 days (Table II).

Importance of age of crab eggs

Attempts made to determine if age of crab eggs was important with respect to feeding rate did not provide clear results. Worms seemed to eat primarily yolk (food reserves), which became only a small portion of the crab eggs as embryos approached hatching. It is possible, although not proven by this study, that decreasing yolk in normal host eggs is the cue that initiates decreased feeding and worm shrinkage after worm reproduction. Several examples suggest the above: a male from *H. oregonensis* ate 18 eggs in 23 days, then 0 near-hatching eggs in the next 8 days, but ate 4 younger eggs in the next 11 days. A female from *H. oregonensis* ate 32 eggs in 17 days, then 2 near-hatching eggs in 14 days, then 12 younger eggs in the next 11 days. In an experiment with four post-reproductive females with near-hatching eggs and four mature-to-post-reproductive females with young crab eggs, the only animal to eat was a post-reproductive female provided young eggs (11 eggs in 25 days). However,

some *C. errans* individuals did eat near-hatching eggs of *H. oregonensis* when those were the only food provided. Some of the differences in how long it took developing females to commence egg laying (Table II, especially for worms from *C. magister*) might be explained by noting that animals in fall and winter were fed different-aged eggs from several female crabs. On the other hand, individuals of *C. errans* in summer and worms from *Cancer antennarius* (Table II) were fed eggs from the same females of *H. oregonensis;* the eggs these worms ate were therefore the same age. Finally, in monitoring the development of worms on a female of *C. magister* that died before her zoeae were released, I found that when development of the crab eggs became arrested prior to the crab's death, reproductive development of her resident worms also ceased.

Egg preference of worms from Hemigrapsus oregonensis *and* Cancer magister

In the few attempts made to determine host egg preference, by putting worms from either *Hemigrapsus oregonensis* or *Cancer magister* into a container with clumps of eggs from both crab species, results showed no strong preference of worms for their own host eggs. Worms were found on, and ate, both types of eggs.

Importance of egg contamination

On several occasions crab eggs used in the experiments became fouled with what appeared to be filamentous fungi. The typical behavior of worms with fouled eggs was to decrease or stop feeding, and crawl as far from the egg clump as possible. In a few cases, worms from *H. oregonensis* actually died in such conditions. When fouled eggs were exchanged for healthy ones, worms would usually return to feeding. However, the hiatus in feeding due to fouling may be a major cause for some of the variations in time to first reproduction. Examples include worms from *H. oregonensis* on eggs of *C. magister* (Table II), the difference in fall-winter and summer time to reproduction for *Carcinonemertes errans* (Table II), and at least one experiment during fall with *C. errans* on *H. oregonensis* eggs. Some of the low feeding rates may also reflect problems with egg fouling.

Discussion

These studies indicate that species of *Carcinonemertes* from various hosts will readily feed, grow, and reproduce on their own or alternate crab host eggs. The feeding rates of worms from different hosts were basically similar, when these worms were fed the same species of crab eggs. Likewise, in experiments in which worms were fed eggs of various hosts, the time for worms to mature and lay their first egg strings was more variable within worm species than between worms from different hosts, when both were fed on the same species of eggs. Much greater differences in feeding rate occurred between juveniles and males *versus* females than between worms from different hosts; and the worms from different hosts would probably have even more similar feeding rates and times before the first egg strings were laid if experiments were controlled more carefully with respect to crab egg contamination and age of crab eggs used as food. The worms from different hosts appear to have similar requirements and appear to be quite uniform with respect to basic feeding and developmental rates when they are away from normal host influences. These same worms display markedly different timing in development and egg laying when on their normal hosts. The examples of a 44-day period of feeding, development, and release of larvae in worms on *Hemigrapsus oregonensis* (Kuris, 1978; Roe, 1979) *versus* an 85–90 day period for the same events in *Carcinonemertes errans* on central California *Cancer*

magister (Wickham, 1980) coupled with the present laboratory situations for both these worms, indicate that many of the differences between worms from different hosts are responses to crab environment rather than intrinsic differences in the worms.

Since worms from several hosts had such similar feeding and reproductive rates in these experiments, these rates can probably safely be used as typical for the carcinonemerteans on most brachyuran hosts, at least along the California coast, under laboratory conditions. Hamilton (1984) found similar laboratory feeding rates for *C. errans* on eggs of its normal host, *C. magister*. In addition, these laboratory feeding rates, especially for female worms, are comparable to estimates made by Wickham (1979) of *C. errans* feeding rates in nature. He estimated that individual worms eat an average of 70 eggs during a brooding period of *C. magister*. In these laboratory feeding experiments females of worms from *H. oregonensis* were estimated to eat about 57 eggs, and females of *C. errans* to eat about 68 eggs as they completed their reproductive cycles.

Although worms from various hosts readily ate eggs of other brachyuran species, neither worms from *H. oregonensis* nor *Carcinonemertes errans* attempted to feed on lobster eggs. Lobster eggs may not be attractive to the worms, or they may simply be too large or have too thick or tough membranes for the worms to handle. The eggs of the three species of crabs used were of similar size, eggs of *H. oregonensis* and *C. jordani* being about 0.3–0.35 mm and eggs of *C. magister* being about 0.42 mm in diameter. Lobster eggs were much larger (about 1.7–1.8 mm diameter) than the brachyuran eggs. Fleming and Gibson (1981) recently described *Pseudocarcinonemertes homari* found on the American lobster and thought to eat lobster eggs. *P. homari* has a stylet about twice as long (average 21.2 μm, Fleming and Gibson, 1981) as the stylets of West Coast *Carcinonemertes* used in this study (10.3–11 μm, Wickham, 1978). In addition, the proboscis of *P. homari* is eversible beyond the front end of the worm and is a rather substantial structure, in contrast to the small proboscis of *Carcinonemertes*. Membranes of lobster eggs show thick and thin areas. Thick parts are about 7.5 μm wide, and thinner areas are about 3.8 μm wide, so the stylets of *Carcinonemertes* from brachyuran crabs are longer than the thickness of lobster egg membranes and should be able to penetrate the membranes. However, it is not known whether the *Carcinonemertes* proboscis has the power to effect penetration. Possibly, the worms normally found on brachyuran hosts cannot penetrate the thick, tough lobster egg membrane.

These studies indicate a low level of host specificity in feeding biology of West Coast species of *Carcinonemertes*. Worms from *Cancer magister* and *Hemigrapsus oregonensis* placed into a fingerbowl will also crawl onto and remain on unnatural hosts (Roe, unpubl.). Nothing is known about host selectivity by settling worm larvae, and worms may show much host specificity at that point in their life history. However, if worms do find themselves on unusual hosts, or if host specificity is actually low, they can certainly complete their life history on a variety of hosts.

Carcinonemertes errans occurs in epidemic numbers on *Cancer magister* (Wickham, 1979) and efforts are underway to find methods to control numbers of this worm on this commercially valuable host (Kuris, 1981–1982). If these worms show little host specificity, or if even a small portion find themselves on alternate sympatric hosts, then results from this study would indicate that effective control of the worms will be more complex than previously thought. Similar rationale pertains to the *Carcinonemertes epialti* group found on several potentially commercially valuable species.

These studies also broadened the potential for several types of laboratory studies of *Carcinonemertes*. One or another species of brachyuran crab with ovigerous females can usually be found along the U. S. west coast any time, and since the worms

successfully eat a variety of eggs, several types of experiments will no longer be confined to the sometimes highly seasonal normal host brooding period. In addition, if several "species" of worms are fed on eggs of the same crab host, sources of variability between worms due to food can be eliminated. Such information will be useful in electrophoretic studies of worms as part of determination of the actual number of worm species found on the U. S. west coast (D. E. Wickham, U. C. Davis and Bodega Marine Laboratory, pers. comm.).

A totally unexpected result from these experiments was the observation that worms taken from male crabs and presumed to be sexually immature, and raised in isolation, laid egg strings in which the eggs developed anyway (Roe, pers. obs.). Although the mechanism of producing larvae by single individuals was not determined for the present study, the result nevertheless adds more complexity to the study of the number of worm species. The idea of putting male worms from one host with females of another host to see if they produce viable offspring is a naive, unworkable method to determine species of west coast *Carcinonemertes.*

This research opens avenues of study previously unrecognized as useful, and at the same time, indicates that some problems with respect to the west coast species of *Carcinonemertes* are more complex than previously thought.

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LITERATURE CITED

COE, W. R. 1902. The nemertean parasites of crabs. *Am. Nat.* **36:** 431–450.

CROWE, J. H., L. M. CROWE, P. ROE, AND D. E. WICKHAM. 1982. Uptake of DOM by nemertean worms: association of worms with arthrodial membranes. *Am. Zool.* **22:** 671–682.

FLEMING, L. C., AND R. GIBSON. 1981. A new genus and species of monostiliferous hoplonemerteans, ectohabitant on lobsters. *J. Exp. Mar. Biol. Ecol.* **52:** 79–93.

HAMILTON, S. A. L. 1984. Feeding rates and growth of *Carcinonemertes errans.* MS Thesis, California State College Stanislaus, Turlock, California.

HUMES, A. G. 1942. The morphology, taxonomy, and bionomics of the nemertean genus *Carcinonemertes. Ill. Biol. Monogr.* **18:** 1–105.

KURIS, A. M. 1978. Life cycle, distribution and abundance of *Carcinonemertes epialti,* a nemertean egg predator of the shore crab, *Hemigrapsus oregonensis,* in relation to host size, reproduction and molt cycle. *Biol. Bull.* **154:** 121–137.

KURIS, A. M. 1981–1982. *Effect of Nemertean Egg Predators on the Dungeness Crab Fishery.* A proposal submitted for 1981–1982 California Sea Grant College Program Support.

MCDERMOTT, J. J. 1976. Observations on the food and feeding behavior of estuarine nemertean worms belonging to the order Hoplonemertea. *Biol. Bull.* **150:** 57–68.

ROE, P. 1979. Aspects of development and occurrence of *Carcinonemertes epialti* (Nemertea) from shore crabs in Monterey Bay California. *Biol. Bull.* **156:** 130–140.

ROE, P., J. H. CROWE, L. M. CROWE, AND D. E. WICKHAM. 1981. Uptake of amino acids by juveniles of *Carcinonemertes errans* (Nemertea). *Comp. Biochem. Physiol.* **69A:** 423–427.

STRICKER, S. A., AND C. G. REED. 1981. Larval morphology of the nemertean *Carcinonemertes epialti* (Nemertea: Hoplonemertea). *J. Morphol.* **169:** 61–70.

WICKHAM, D. E. 1978. A new species of *Carcinonemertes* (Nemertea: Carcinonemertidae) with notes on the genus from the Pacific coast. *Proc. Biol. Soc. Wash.* **91:** 197–202.

WICKHAM, D. E. 1979. Predation by the nemertean *Carcinonemertes errans* on eggs of the dungeness crab *Cancer magister. Mar. Biol.* **55:** 45–53.

WICKHAM, D. E. 1980. Aspects of the life history of *Carcinonemertes errans* (Nemertea: Carcinonemertidae), an egg predator of the crab *Cancer magister. Biol. Bull.* **159:** 247–257.

Reference: *Biol. Bull.* **167**: 437–444. (October, 1984)

MORPHOLOGICAL OBSERVATIONS AND MICROSPECTROPHOTOMETRIC DATA FROM PHOTORECEPTORS IN THE RETINA OF THE SEA RAVEN, *HEMITRIPTERUS AMERICANUS*

B. A. COLLINS* AND E. F. MACNICHOL, JR.*#

**Laboratory of Sensory Physiology, Marine Biological Laboratory, Woods Hole, Massachusetts 02543, and #Department of Physiology, Boston University School of Medicine, Boston, Massachusetts 02118*

ABSTRACT

A histological survey of the retinas of some fishes has revealed an unusual cone formation. We repeatedly find these unusual triple cones in 10% of the retinas reviewed. To obtain further information about these photoreceptors, the sea raven, whose retina is known to contain them, was chosen for further study. Microspectrophotometric measurements were made to determine the peak absorbance of the visual pigment contained in the three individual outer segments. We report here the results of measurements made on triple cones found in the retina of the sea raven, *Hemitripterus americanus*.

INTRODUCTION

In a continuing histological survey of the retinas of fishes indigenous to the Woods Hole, Massachusetts area, numerous preparations are reviewed (Collins and MacNichol, 1978). An unusual cone type, a triple cone (Vrabec, 1955; Ali and Anctil, 1976) appears in approximately 10% of the fish retinas prepared (Collins and MacNichol, 1979). Its development and function is not known. Most of the fish species studied have two morphologically different cone types, double cones and single cones. Microspectrophotometric data on these cone types indicates that the paired outer segments of the double cones usually contain either two long-wave ("red-red") or two middle-wave ("green-green") visual pigments; or one of the outer segments contains the long-wave, the other the middle-wave pigment ("red-green"). The outer segment of the smaller single cone usually bears the short-wave ("blue") visual pigment (Stell and Hárosi, 1976; Levine and MacNichol, 1979; Hárosi, 1982).

Triple cones are approximately the same size as double cones and, indeed, can be easily overlooked. We first saw these triple cones in histological preparations which were made by sectioning tangentially at the ellipsoid level of the retina. Once it became apparent that this unusual cone formation was not artifactual, it was decided to obtain further information about these interesting photoreceptors.

Since the histological preparations were made from randomly chosen tissue throughout the retina of the sea raven, it was not known where the triple cones would be located. Also, having made a great number of fresh and partially fixed (Levine *et al.*, 1981) retinal preparations from many of these fish, it appeared that these unusual cones were not in every individual studied. Therefore, we considered these photoreceptors quite rare.

Hárosi (1982) reported preliminary microspectrophotometric (MSP) data on the visual pigments from one triple cone found in the retina of the sea raven, *Hemitripterus*

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americanus. All three outer segments of this triad contained the same green-sensitive pigment. His MSP results and our earlier data, showed that the double cones of the sea raven can either bear the same ("green-green") or different ("red-green") visual pigments. The present investigation was undertaken in an attempt to determine, if indeed, some of the triple cones contain different visual pigments in their outer segments.

Materials and Methods

Sea raven were collected from the waters of the Woods Hole area by research vessels of the National Marine Fisheries Service, Northeast Fisheries Center or by Marine Biological Laboratory vessels. Since sea raven prefer cool, deep waters (Bigelow and Schroeder, 1953), they were best caught in the late fall and throughout the winter season. Many died when they were hauled up from the deep, and those that survived died in our running sea water tanks if the water temperature rose above 55°F. All fish used in this study were more than 8 inches in body length, with some over 15 inches long. No attempt was made to determine their age or sex.

Each sea raven was dark-adapted for at least two hours in oxygenated sea water in a light-tight tank, then anesthetized with tricaine methanesulfonate. Under infrared light the eye was enucleated and hemisected, then the eye cup was placed in a teleost Ringer's solution with the following composition: NaCl (133.5 *M*m), KCl (3.4 *M*m), $MgCl_2$ (9.8 *M*m), NaH_2PO_4 (2.9 *M*m), $NaHCO_3$ (11.9 *M*m), $CaCl_2$ (1.5 *M*m), glucose (11.1 *M*m) with the pH adjusted to 7.8 (this yields approximately 330 mOs/kg). The pigment epithelium separates readily from the sensory retina in 15–30 minutes after immersion in the Ringer's solution. A small piece (*ca.* 1 mm^2) of retinal tissue was cut and, using a pipette, was transferred in a drop of the Ringer's solution to a coverslip. The tissue was surrounded by a ring of silicone oil (Dow Corning #702), and another coverslip was laid carefully on top of the tissue (MacNichol *et al.*, 1978). This coverslip sandwich was transferred to the microscope of the photon-counting microspectrophotometer (PMSP) (MacNichol, 1978). With the aid of the infrared video monitor attached to the PMSP, the preparation was scanned to locate the various cone types. Once a triple cone was located, transverse measurements were made through the outer segments and the visual pigment spectrum was recorded (Stell and Hárosi, 1976; Levine and MacNichol, 1979; Hárosi, 1982; MacNichol *et al.*, 1983). After these measurements were made, the preparation was transferred to a Nomarski differential interference-contrast microscope and an attempt was made to locate and photograph the same triple cone with a Polaroid camera attached to the microscope.

Results

Histological observations of tangentially sectioned preparations through the ellipsoid region of the retina of the sea raven show that photoreceptors form a regular mosaic pattern. As found in many teleosts, four double cones form a square with a single cone in the center (Ali and Anctil, 1976; Kunz, 1980) (Fig. 1a). However, occasionally a triple cone would appear randomly placed within this mosaic pattern (Fig. 1b, c).

In sea raven the two members of the double cones are equal in size, having inner segments approximately 22–25 μm long and 12–15 μm wide, with outer segments about 15 μm long by 3–5 μm wide. Because the three members of the triple cones

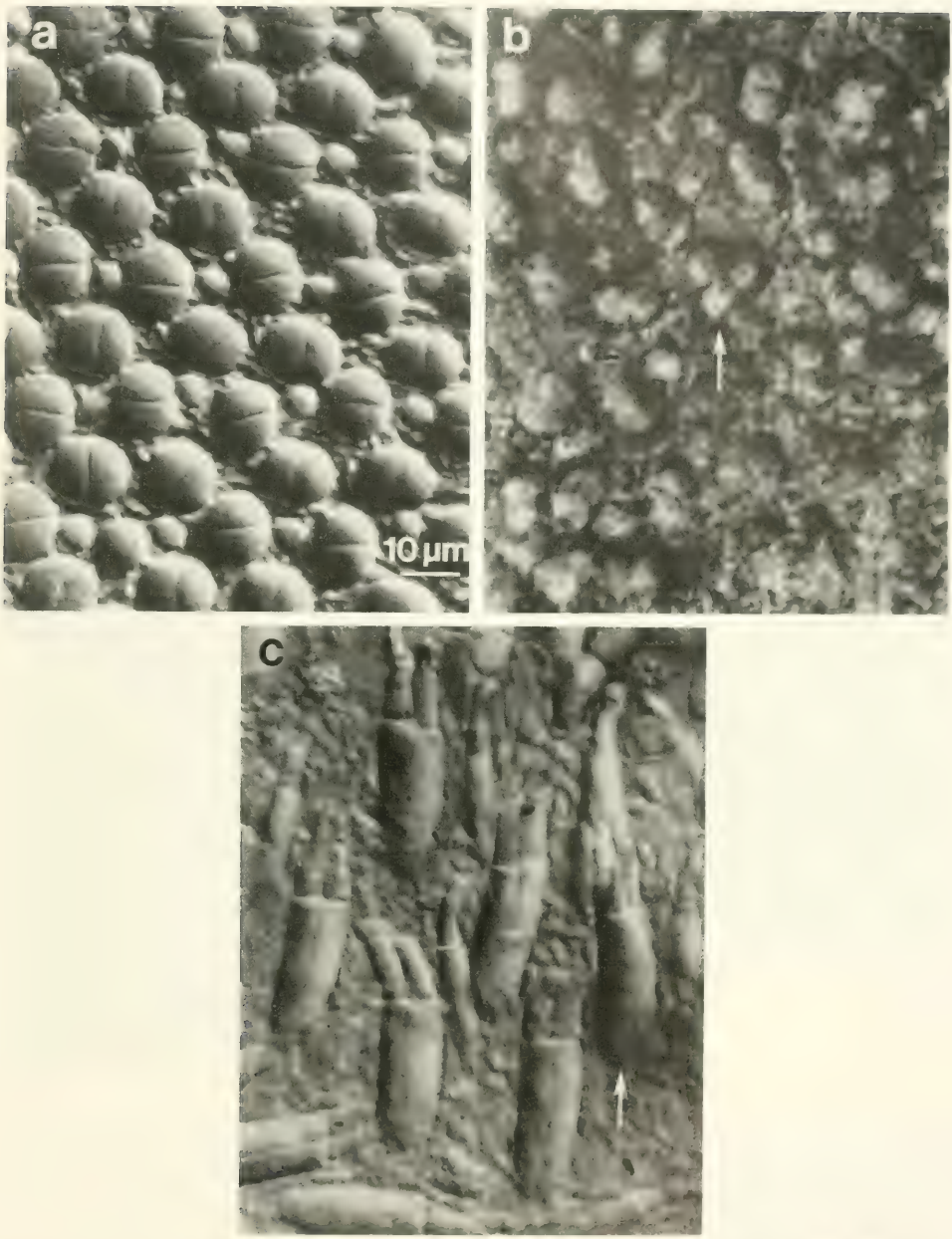

FIGURE 1. Mosaic patterns of the retina of the sea raven. (a) A photomicrograph of a histological preparation of sea raven retina. The preparation was made by sectioning tangentially through the ellipsoid region. A square mosaic pattern is formed by the double and single cones. The photograph was taken using a Polaroid camera attached to a Nomarski interference-contrast microscope. The microscope objective was 40×, the camera eyepiece was 12.5×. (b) Photomicrograph of a fresh whole-mount preparation from the sea raven retina. A view at the ellipsoid level which shows a triple cone appearing among the double and single cones. Arrow points to triple cone. Magnification is the same as in Figure 1a. (c) A photomicrograph of a partially fixed retinal preparation showing double cones that form the square pattern around the single cones of the sea raven. An arrow points to the triple cone that can be seen randomly placed in the mosaic pattern. Magnification is the same as in Figure 1a.

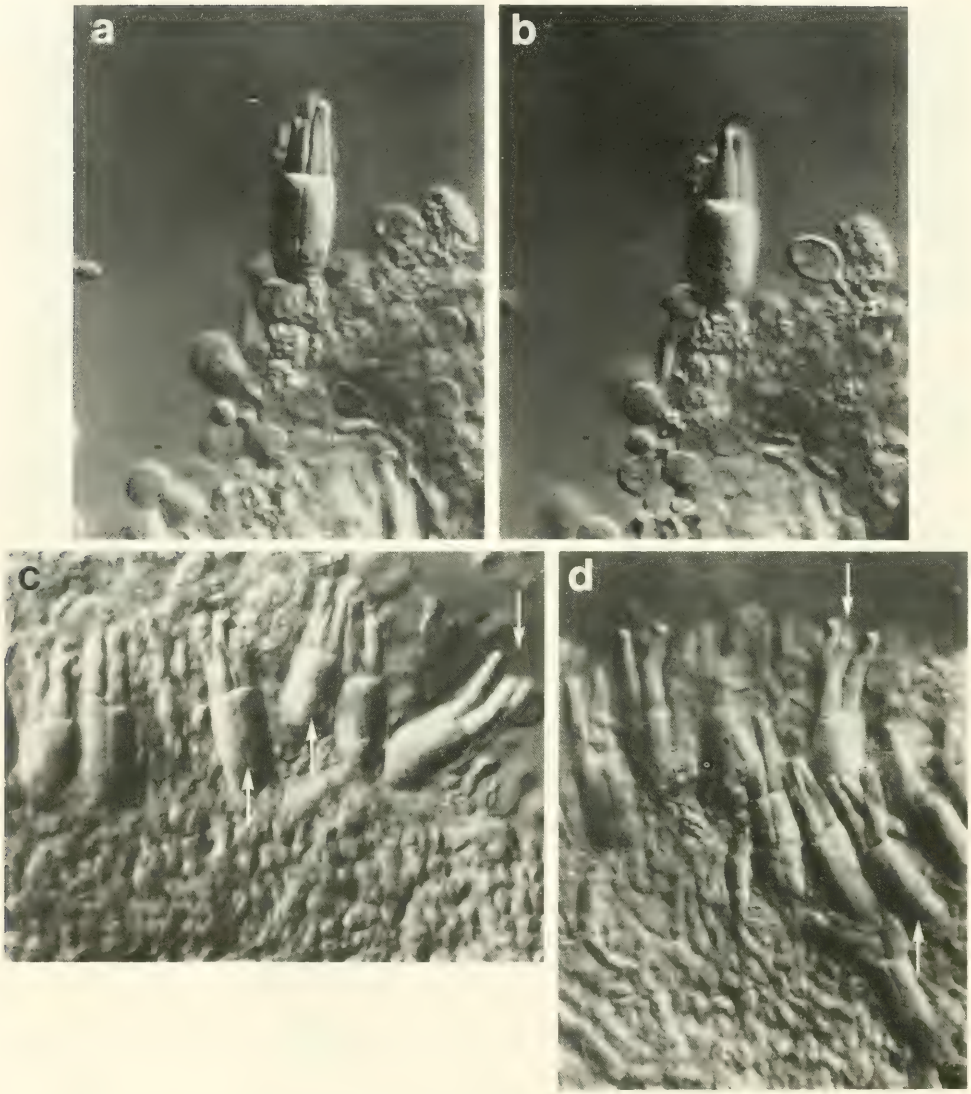

FIGURE 2. Triple cones from the retina of the sea raven. (a) Photomicrograph of an isolated triple cone from partially fixed retinal tissue of the sea raven. The magnification is the same as in Figure 1a. (b) Photomicrograph of the same cone as in Figure 2a. Microscope focus changed slightly bringing the foreground area, and the third member of the triple cone, into focus. Same magnification as Figure 1a. (c) (d) Photomicrographs of clusters of triple cones, interspaced among double and single cones, found in a partially fixed piece of retinal tissue from the sea raven. Arrows point to triple cones. Magnification as in Figure 1a.

are so similar, and most often two of the three outer segments are over-lying, they could easily be mistaken for double cones (Fig. 2a, b). However, once a triple cone was located, several more would often be seen in the vicinity (Fig. 2c, d). The inner segment of the single cone is approximately 12 μm long and 6 μm wide. The outer

segment of the single cone is approximately 15 μm long and 3–5 μm wide, corresponding in size to the double cone.

The sea raven has large photoreceptors, and the comparative ease of enucleation and dissection of the retina makes it an ideal model for numerous visual studies. During various seasons over a period of several years, many sea ravens have been used in our laboratory. Whenever PMSP measurements were made on the retina of this fish, we looked for triple cones (Fig. 3a, b). During this time the outer segments of sixteen triple cones were measured from five different individuals although this cone type was seen in preparations made from the many sea raven retinas reviewed. If the patch of retinal tissue being examined by PMSP contained triple cones, usually only one or two were free from surrounding tissue. Too often, none of the outer segments of the triple cones were optically isolated enough to be measured. From one fish, however, a piece of the retinal tissue yielded seven measurable triples. Occasionally PMSP recordings were made through two over-lying outer segments; this increased the optical density, but the wavelength of peak absorbance (λ_{max}) and half band-width was the same as measurements taken from a single, isolated outer segment of a triple cone if the outer segments bore the same visual pigments. Pigment measurements were accepted according to the criteria of MacNichol *et al.* (1983).

When obtaining PMSP measurements from the double cones, it was noted that the percentage of "green-green" pairs was considerably higher than the "red-green" absorbing pair of outer segments of these cones. From a total of 70 acceptable records, the λ_{max} of 58 green-absorbing cone outer-segments was 521.7 $\pm$ 3.28 nm (S.D.) and the λ_{max} of the 12 red-absorbing cone outer-segments was 560.1 $\pm$ 5.25 nm (S.D.). All 22 single cones measured contained a blue-absorbing pigment with a λ_{max} at 462.5 $\pm$ 3.72 nm (S.D.); the λ_{max} of the pigment in the numerous rods was 505 $\pm$ 2.87 (S.D.). These results are in close agreement with the findings of Hárosi (1982). In fourteen triple cones, all three outer segments contained the same green-sensitive visual pigment (Fig. 3c). Only three of these triple cones yielded data that were acceptable; but from these, the λ_{max} was determined to be 522.1 $\pm$ 5.56 nm (S.D.). However the outer segments of two triple cones contained different visual pigments. PMSP results indicated that two of the three outer segments were green-absorbing and the third bore the red-absorbing pigment. From one of these cones, PMSP scans were taken through the three individual outer segments (Fig. 3d). The better record was made of the second triple cone (Fig. 3e) whose two green-sensitive outer segments were overlapping, yielding an optical density of 0.112 OD which was nearly double that of the third, red-sensitive member (0.061 OD).

Discussion

Histological studies of the retina indicate that sea raven possess a well developed visual system; photopigment data suggests that this fish has the physical capacity to discriminate colors. Such a complex visual system might be unnecessary if this fish remained in the dim narrow-band illumination of the deep water for its entire life cycle. The sea raven is normally found down to 50 fathoms, but it has been taken as deep as 105 fathoms (Bigelow and Schroeder, 1953). Deep water transmits only a narrow blue-green band of the visible spectrum. Receptors having sensitivities outside this range might be of little use, unless they are used to observe bioluminescence which is not present in the raven's normal habitat.

For most of the year adult sea ravens vary in color from reddish purple to chocolate or gray with a yellowish belly. During the breeding season they move to shallow

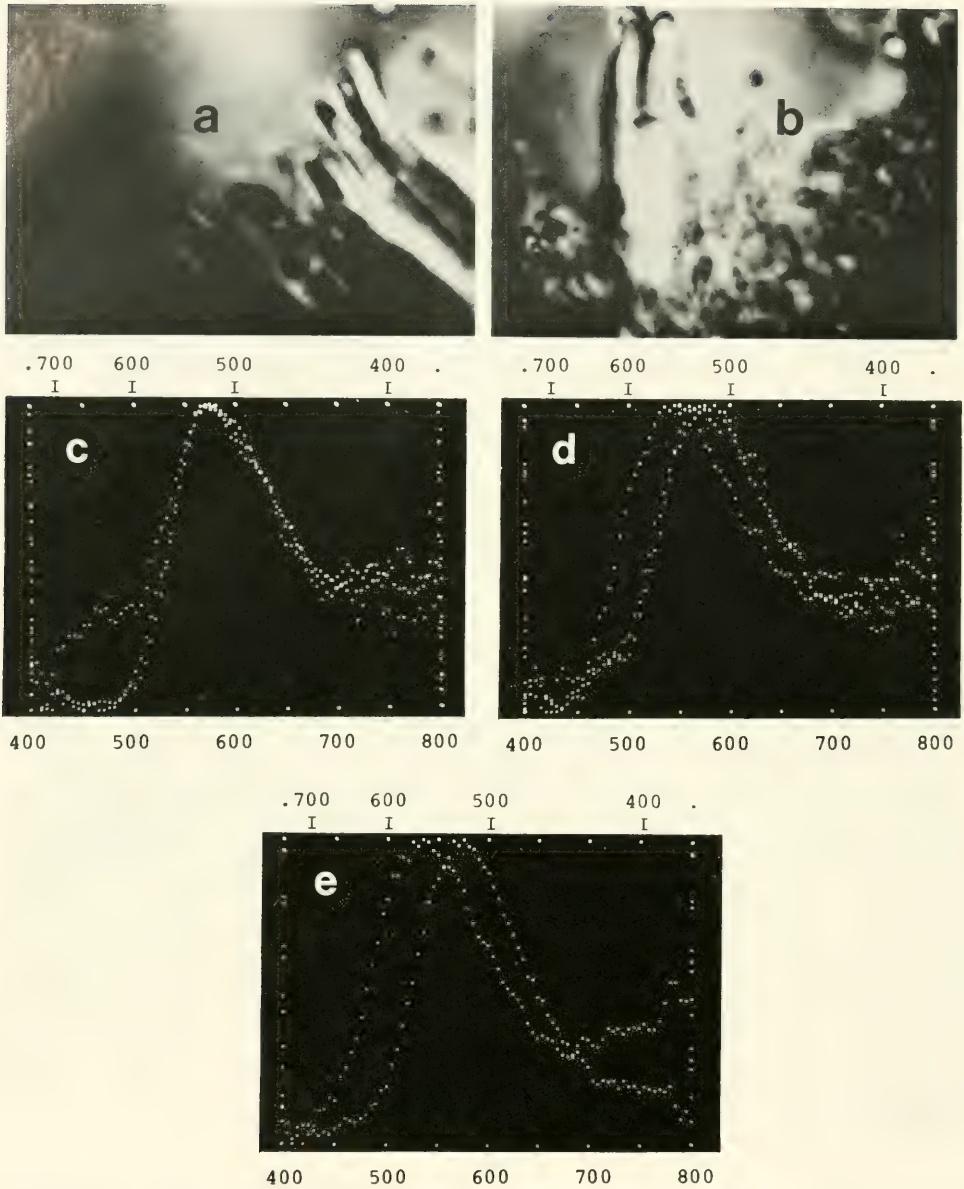

FIGURE 3. Triple cones photographed from the infrared video monitor of the PMSP at the time of measurement, and the records of some of the measurements obtained. (a) Note the white spot in the center member of the three outer segments illustrated in this photograph; this is the measuring beam of the PMSP. The magnification is slightly higher than in Figure 1a. (b) The three outer segments of the triple cone are tightly clumped together making individual PMSP measurements difficult to obtain. The magnification is the same as in Figure 3a. (c) Superimposed records of the pigment absorbance spectra from all three outer segments of a triple cone found in the retina of the sea raven. This record indicates that each outer segment bears the same green-absorbing pigment. The measured values of λ_{max} are: 519, 519, and 523 nm. (d) Superimposed records of the pigment absorbance spectra from the three outer segments of another triple cone showing that one of the members is red-absorbing while the other two are green-absorbing. (Measured λ_{max} = 554, 524, and 524 nm.) (e) Superimposed records from a second triple cone bearing one red-absorbing member. One scan was made through the red-absorbing outer segment. The other was made with the beam passing in succession through both green-absorbing outer segments since they were overlapping; this yielded nearly double the optical density of the first member. (Measured λ_{max} = 565 and 525 nm.) All records are displayed on a linear frequency scale indicated in Terahertz by the numbers at the bottom. The upper non-linear scale is in nanometers. (Thz = 100,000/nm = 10^{12} Hz). The individual points are at 5 THz intervals and are derived from total photon counts in the 5 THz region surrounding each point.

water where a broad spectrum of illumination is available, and assume a brillant body coloration, from bright yellow through orange to blood-red (Goode, 1888). In southern New England, from early October to late December, the sticky egg clusters of the sea raven are deposited on the branches of the pinkish finger sponge, *Chalina,* or on the yellow breadcrumb sponge, *Halichondria* (Warfel and Merriman, 1944). A highly developed ability to make visual discriminations at this critical part of the life cycle could be an important advantage in recognition of both a suitable mate and suitable area for egg deposition.

McFarland and Munz (1975) measured the characteristics of the photic environment of the surface, middle, and bottom dwelling fishes. Microspectrophotometric measurements of the visual pigments of some of these fishes suggest that color sensitivity evolved by a fish species may be related to the photic environment in which it lives (Levine and MacNichol, 1979). For example, surface dwelling fishes are sensitive throughout the visible spectrum, indeed some have photoreceptors sensitive into the ultraviolet (Hárosi and Hashimoto, 1983), whereas the sensitivity of those fishes dwelling near the bottom is greatest in the blue-green region of the spectrum.

The behavior of the sea raven indicates that it ranges over a variety of habitats with varying photic environments. The microspectrophotometric measurements of the photopigments of this fish suggests that its visual system is capable of coping with these environmental changes. The sea raven lives in deep water for most of the year. At this time perhaps the triple cone, increasing the visual sensitivity in the blue-green region, becomes extremely important for the survival of this species. Whereas during the three month breeding season, the long-wavelength region of the raven's visual system is utilized for the all important reproduction cycle and its egg deposition.

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LITERATURE CITED

ALI, M. A., AND M. ANCTIL. 1976. *Retinas of Fishes, An Atlas.* Springer-Verlag, Berlin, Heidelberg, and New York. 284 pp.

BIGELOW, H. B., AND W. C. SCHROEDER. 1953. *Fishes of the Gulf of Maine,* Fishery Bull. Fish. Wild. Serv. U. S. **74**(53): 454–457.

COLLINS, B. A., AND E. F. MACNICHOL, JR. 1978. Long term fixation for histological studies. *Microsc. Acta* **81**(2): 155–158.

COLLINS, B. A., AND E. F. MACNICHOL, JR. 1979. Triple cones found in the retinas of 3 fish species. *Experientia* **35**: 106.

GOODE, G. BROWN. 1888. P. 302 in *American Fishes.* W. A. Houghton, New York.

HÁROSI, F. I. 1982. Recent results from single-cell microspectrophotometry: cone pigments in frog, fish and monkey. *Color Res. Application* **7**(2, part 2): 135–141.

HÁROSI, F. I., AND Y. HASHIMOTO. 1983. Ultraviolet visual pigments in a vertebrate: A tetrachromatic cone system in the dace. *Science* **222**: 1021–1023.

KUNZ, Y. W. 1980. Cone mosaics in the teleost retina: Changes during light and dark adaptation. *Experientia* **36**: 1371–1374.

LEVINE, J. S., AND E. F. MACNICHOL, JR. 1979. Visual pigments in teleost fishes: effect of habitat, microhabitat and behavior on visual system evolution. *Sensory Proc.* **3**: 95–131.

LEVINE, J. S., S. L. GOODMAN, AND E. F. MACNICHOL, JR. 1981. Cone pigments: Aldehyde fixation for microspectrophotometry. *Invest. Ophthal. Vis. Sci.* (Abstr.) 104.

MacNichol, E. F., Jr. 1978. A photon-counting microspectrophotometer for the study of single-vertebrate cells. Pp. 194–208 in *Frontiers in Visual Science,* S. J. Cool and E. L. Smith, eds. Springer-Verlag, New York.

MacNichol, E. F., Jr., Y. W. Kunz, J. S. Levine, F. I. Hárosi, and B. A. Collins. 1978. Ellipsosomes: Organelles containing a cytochrome-like pigment in the retinal cones of certain fishes. *Science* **200:** 549.

MacNichol, E. F., Jr., J. S. Levine, R. J. W. Mansfield, L. E. Lipetz, and B. A. Collins. 1983. Microspectrophotometry of visual pigments in primate photoreceptors. Pp. 13–38 in *Colour Vision: Physiology and Psychophysics.* J. D. Mollon and L. T. Sharpe, eds. Academic Press, London.

McFarland, W., and F. W. Munz. 1975. Part II: The photic environment of clear tropical seas during the day. *Vision Res.* **15:** 1063–1070.

Stell, W. K., and F. I. Hárosi. 1976. Cone structure and visual pigment content in the retina of the goldfish. *Vision Res.* **16:** 647–657.

Vrabec, F. 1955. Sur les cônes triples de la rétine de téléostéens. *Vistnik Cesk. Spol. Zool.* **19**(2): 183–186.

Warfel, H. E., and D. Merriman. 1944. The spawning habits, eggs and larvae of the sea raven, *Hemitripterus americanus,* in southern New England. *Copeia* **4:** 197–205.

Reference: *Biol. Bull.* **167**: 445–459. (October, 1984)

CHEMOAUTOTROPHIC SYMBIONTS IN THE BIVALVE *LUCINA FLORIDANA* FROM SEAGRASS BEDS

MARK R. FISHER AND STEVEN C. HAND[1]

Department of Biology, University of Southwestern Louisiana, P.O. Box 42451, Lafayette, Louisiana 70504

ABSTRACT

Enzymatic and histological evidence suggest that the eulamellibranch bivalve *Lucina floridana* possesses bacterial endosymbionts capable of a chemoautotrophic metabolism. Dense populations of *L. floridana* (83 ± 11 per m^2; 95% CI, n = 33) are found closely associated with the O_2-releasing root systems of seagrasses in sulfide-rich sediments; the sandy sediments of both *Thalassia* and *Ruppia* beds contain 1.67 ± 0.31 m*M* (95% CI, n = 13) and 2.49 ± 0.55 m*M* (95% CI, n = 13) sulfide, respectively. Both transmission electron microscopy of gill tissue and scanning electron microscopy of freeze-fractured gills reveal numerous rod-shaped procaryotic inclusions in vacuoles of large, eucaryotic cells ("bacteriocytes") located deeply within demibranch cross sections; no such inclusions are seen in the ciliated gill epithelium which is rich in mitochondria. Activities of ribulose 1,5-bisphosphate carboxylase (RuBPCase), phosphoribulokinase, APS reductase, ATP sulfurylase, and nitrite reductase have been measured and partially characterized in homogenates of fresh gill tissue. Light microscopy reveals numerous aggregations of pigmented granules localized to the interior of the gill in association with the bacteriocytes. Histochemical staining demonstrates the presence of iron in these granules, consistent with the idea that their composition, in part, may be respiratory pigment and/or iron-containing cytochromes. Energy dispersive X-ray analysis reveals sulfur as a dominant inorganic element in the gill tissue. Based on abundance data of *L. floridana* and *in vitro* levels of RuBPCase (half-maximal velocity) this bivalve could potentially contribute 336 ± 96 g C/m^2/year (95% CI) to the gross carbon fixation of seagrass beds.

INTRODUCTION

Seagrass beds are now recognized as one of the most productive of marine communities, with primary production values of 500–1000 g C/m^2/year; high production areas may reach over twice this figure (Fenchel, 1977; Zieman and Wetzel, 1980). Since a large portion of the plant material produced is eventually deposited on the bottom as detritus (Kikuchi and Peres, 1977), the abundance of organic material in the sediment is often greater than the oxygen available for its degradation. Under these anaerobic conditions, decomposition is accomplished by the activity of certain bacteria which can utilize inorganic compounds other than O_2 as electron acceptors; $SO_4^=$, NO_3^-, CO_2, and H_2O may be reduced to H_2S, NH_3, CH_4, and H_2 (Fenchel and Riedl, 1970). Hydrogen sulfide is often the major inorganic constituent in the sediments due to the high availability of sulfates in sea water and the large populations of sulfate-reducing bacteria, such as *Desulfovibrio* sp. (Jorgensen and Fenchel, 1974).

Normally, metazoans are poorly represented in this sulfide biome, the predominant

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[1] To whom reprint requests should be addressed.

members being facultative/obligate anaerobes from the platyhelminthes and the aschelminthes (Fenchel and Riedl, 1970). However, oxygen is known to diffuse from the roots of marine angiosperms (Teal and Kanwisher, 1966; Armstrong, 1970) forming aerobic zones in the immediate vicinity of the root systems (oxidized rhizospheres), and it is among the roots of *Thalassia* and *Ruppia,* two seagrasses common to high-salinity, coastal waters of the Gulf of Mexico (Edwards, 1976), that the eulamellibranch bivalve *Lucina floridana* is known to occur (Britton, 1970). This habitat would potentially provide this bivalve with simultaneous access to both oxygen and hydrogen sulfide. Such an interface between these two molecular species is very similar to that documented for the effluent waters of the hydrothermal vents along the Eastern Pacific Rise (*e.g.,* see Edmond *et al.,* 1982). Consequently, we chose to study selected enzyme systems and the gill ultrastructure of the shallow-living bivalve *L. floridana* to determine, first, if the potential for chemoautotrophic metabolism was present in this species, as was described for certain hydrothermal vent organisms (Cavanaugh *et al.,* 1981; Felbeck, 1981; Felbeck *et al.,* 1981; Felbeck and Somero, 1982; Cavanaugh, 1983). The vestimentiferan tube worm *Riftia pachyptila,* the clam *Calyptogena magnifica,* and the unidentified vent mussel possess symbiotic sulfur-oxidizing bacteria capable of a chemoautotrophic metabolism. More recently, species of non-hydrothermal vent bivalves have also been identified as having sulfur-based metabolism (Cavanaugh, 1983; Felbeck, 1983; Berg and Alatalo, 1984). Second, clarifying the spatial arrangement and position of the endosymbionts relative to other tissue structures (*e.g.,* the distinctive pigment granules of lucinid gills) could indicate the degree of access of molecular species like oxygen and sulfide to the bacteria. Finally, such a chemoautotrophic metabolism, if present in *L. floridana,* could represent a significant amount of gross carbon assimilation, which has as yet been unappreciated for seagrass ecosystems.

Materials and Methods

Specimen collection and population densities

Specimens of *L. floridana* were collected from the turtle grass (*Thalassia testudinum*) and widgeon grass (*Ruppia maritima*) beds along the western shore of St. Joseph's Bay, Florida, in 0.25–1.0 m of water, and from the widgeon grass beds along the mainland shore of Alligator Harbor, Florida at a depth of 1–2 m. Specimens were maintained in their original substrate (sulfide rich) in 20 gallon aquaria at 32 ppt salinity. All experimental animals were used within three weeks of collection, although they routinely survived several months under the above conditions in the laboratory. Population densities at the St. Joseph's Bay site were measured with a 0.1 m^2 quadrat thrown from three separate transects at intervals of 30, 45, 60, 75, and 100 meters from shore. All specimens were collected from a sediment depth of 0–20 cm; no specimens were found below 20 cm.

Sulfide measurements

Sulfide concentrations were determined in sediments at depths of 5, 10, 15, and 20 cm in both *Thalassia* and *Ruppia* beds, using sections sliced from core samples. Sediment samples were diluted 1:1 (w/v) with a sulfide antioxidant buffer consisting of 80 g NaOH, 320 g sodium salicylate, and 72 g ascorbic acid in 1000 ml distilled water (modified after Baumann, 1974). All samples were voided of air bubbles and kept on ice in airtight bags. Sulfide measurements were made within 36 hours with an Iotrode AB 120 sulfide electrode and an Orion 407A specific ion meter. The amount of interstitial water was analyzed by drying a fresh sediment sample of known

weight at 90°C for 36 hours. The difference between wet and dry weights was then used as a measure of interstitial water.

Enzymatic activity determinations

All enzymatic assays were performed using fresh gill tissue which was homogenized in three volumes of the appropriate, ice-cold medium with a Wheaton ground glass homogenizer. The crude homogenates were centrifuged at 5000 × *g* for 15 minutes and the supernatants were saved and used for the assays without further purification.

Ribulose 1,5-bisphosphate carboxylase [3-phospho-D-glycerate carboxylase (dimerizing), EC 4.1.1.39] activity was measured using the protocol as described by Wishnick and Lane (1971). The homogenizing medium consisted of 10 m*M* tris-HCl buffer (pH 7.8), 0.1 m*M* EDTA, and 10 m*M* β-mercaptoethanol. The 0.5 ml reaction mixture contained 100 μmoles tris-HCl buffer (pH 7.6), 5 μmoles $MgCl_2$, 25 μmoles $NaHCO_3$, 0.03 μmoles EDTA, 3.0 μmoles glutathione, 1.0 μmole ribulose 1,5-bisphosphate, and 2 μCi of $NaH^{14}CO_3$ (specific activity, 50 μCi/μmole). The reaction mixture was incubated for 10 minutes at 20°C, stopped with 6 *N* HCl, and heated at 90°C for 60 minutes. Radioactivity incorporated into the acidified sample was measured by liquid scintillation counting.

The method of Racker (1975) was followed for the assay for phosphoribulokinase (ATP:D-ribulose-5-phosphate 1-phosphotransferase, EC 2.7.1.19). Gill tissue was homogenized in 1.0 *M* tris-HCl buffer (pH 7.8). The assay mixture contained 50 μmoles tris-HCl buffer (pH 7.8), 10 μmoles $MgCl_2$, 10 μmoles glutathione, 12 μmoles ATP, 75 μmoles $KHCO_3$, 0.15 μmoles NADPH, 5 μmoles ribose-5-phosphate, 5 units ribose-5-phosphate isomerase, 5 units glyceraldehyde-3-phosphate dehydrogenase, and 5 units phosphoglycerate kinase in a final volume of 1.0 ml. The oxidation of NADPH was followed at 340 nm.

The method described by Peck *et al.* (1965) was used for the assay of APS reductase [AMP, sulfite:(acceptor) oxidoreductase, EC 1.8.99.2]. The gill tissue was homogenized in 0.3 *M* tris-HCl buffer (pH 8.0) and 5 m*M* EDTA. The 3.0 ml reaction mixture contained 300 μmoles tris-HCl buffer (pH 8.0), 10 μmoles AMP, 4 μmoles sodium ferricyanide, and 10 μmoles sodium sulfite. The reduction of the ferricyanide ion was monitored by measuring the decrease in absorbance at 410 nm (λ max). The millimolar extinction coefficient of oxidized ferricyanide in distilled water was empirically determined to be 0.927 millimoles^{-1} cm^{-1} at 410 nm. Background rates of the reaction mixture without AMP were subtracted to correct for sulfite oxidase activity.

ATP sulfurylase (ATP:sulfate adenyltransferase, EC 2.7.7.4) was assayed according to the method of Felbeck (1981). The homogenizing medium consisted of 0.1 *M* triethanolamine-HCl buffer (pH 7.3). The assay medium consisted of 0.1 *M* triethanolamine-HCl buffer (pH 7.3), 2.5 μmoles magnesium acetate, 25 μmoles glucose, 25 μmoles pyrophosphate, 0.05 μmoles $NADP^+$, 0.05 μmoles adenosine phosphosulfate, 2.5 units hexokinase, 1.25 units glucose-6-phosphate dehydrogenase, and 1 μmole P^1, P^5-di(adenosine-5′) pentaphosphate in a total volume of 2 mls. The reduction of $NADP^+$ was followed at 340 nm.

Rhodanese (thiosulfate:cyanide sulfurtransferase, EC 2.8.8.1) activity was assayed following the method of Smith and Lascelles (1966). Gill tissues were homogenized in 0.3 *M* tris-HCl buffer (pH 8.7). The 3 ml reaction mixture consisted of 300 μmoles tris-HCl buffer (pH 8.7), 150 μmoles NaS_2O_3, 200 μmoles half-neutralized sodium cyanide (NaCN:HCl, 2.5:1 molar ratio), 0.5 μmoles 2,6-dichlorophenol-indophenol (DCIP), and 25 mg phenazine methosulfate (PMS). The reduction of DCIP was followed at 600 nm.

Finally, nitrite reductase (ammonia:ferredoxin oxidoreductase, EC 1.7.7.1) activity was determined by the procedure described by Losada and Paneque (1971). The homogenizing medium consisted of 0.5 *M* tris-HCl buffer (pH 8.0). Each reaction flask contained, in a volume of 2 mls, 150 μmoles tris-HCl buffer (pH 8.0), 4 μmoles $NaNO_2$, 1.5 μmoles methyl viologen, and 44 μmoles sodium dithionite, added from a stock solution prepared in 0.29 *M* $NaHCO_3$. The reaction mixture was incubated for 30 minutes at 20°C, and the amount of substrate remaining in each reaction flask was determined colorimetrically (Losada and Paneque, 1971).

Protein analysis

For all gill homogenates the protein concentration in the supernatant was measured using the technique of Peterson (1977).

Scanning electron microscopy

Gill tissues were fixed with 5% glutaraldehyde in 0.1 *M* phosphate buffer (pH 7.4) to which sucrose was added to bring the final osmolality to 1200 mOsm/kg. Postfixation was carried in 0.1 *M* phosphate buffer (pH 7.4) containing 2% osmium tetroxide, and dehydration was accomplished by a graded acetone series. The dehydrated tissue was immersed in liquid nitrogen and fractured with a precooled razor blade. After critical point drying, the fractured tissues were mounted on a stub and sputter coated with gold-palladium.

Energy dispersive X-ray analysis

Tissues were fixed with 5% glutaraldehyde as above, but were not postfixed or dehydrated using organic solvents. Instead, they were allowed to air dry in a desiccator at room temperature for 48 hours, freeze-fractured as above, mounted, and coated by carbon vapor deposition. Tissues were not critical point dried.

Transmission electron microscopy

Materials were fixed in 0.3 *M* PIPES (pH 7.8) containing 4% paraformaldehyde and 5% glutaraldehyde. The osmolality was adjusted to 1200 mOsm/kg with filtered sea water. Tissues were postfixed with 2% osmium tetroxide in 0.3 *M* PIPES (pH 7.8) and dehydrated in a graded acetone series. Using the rapid infiltration method described by Millonig (1976), tissues were placed in a Spurr's low-viscosity resin/acetone mixture (1:1) on a rotator for 30 minutes and then transferred to 100% Spurr's and centrifuged at 1250 × *g* for 20 minutes. The centrifugation step was repeated once using fresh resin. Specimens were embedded in fresh Spurr's for 18 hours at 50°C, and sections were stained with 5% uranyl acetate and 0.1% lead citrate.

Histochemical staining

Gill tissue to be stained for the presence of iron was fixed with 5% glutaraldehyde in 0.1 *M* phosphate buffer (pH 7.4) and embedded in Spurr's low viscosity resin. Then 0.5 micron sections were prepared and stained for occult iron as described by Read (1962). Briefly, the procedure consisted of placing the sections into a 30% solution of H_2O_2 for two minutes, rinsing in distilled water, and staining the sections in a heated and acidified solution of 0.06 *M* potassium ferricyanide for fifteen minutes. The tissue was then counter-stained in 1% carminic acid after washing in distilled water. Finally, the sections were differentiated in a 4% potassium aluminum sulfate solution for five minutes and thoroughly rinsed in distilled water.

RESULTS

Hydrogen sulfide levels and population densities

Hydrogen sulfide occurs in the interstitial water of the sediments of both *Thalassia* and *Ruppia* beds at concentrations of 1.67 ± 0.31 m*M* (95% CI, n = 13) and 2.49 ± 0.55 m*M* (95% CI, n = 13), respectively. The compound appears to be uniformly distributed throughout these sandy sediments as a function of depth, since HS^- concentrations were not significantly different at depths of 5, 10, 15, or 20 cm (ANOVA, 95% confidence). Based on data collected from both seagrass beds (Table I), *Lucina floridana* exists in population densities of 83 ± 11 per m^2 (95% CI, n = 33). No statistical difference was shown between the densities found in *Thalassia* beds *versus* *Ruppia* beds (ANOVA, 99% confidence). However, live *Lucina* specimens were not observed in study areas entirely devoid of seagrasses, nor did the bivalves occur at sediment depths greater than 20 cm. This sediment depth corresponds to the maximum depth of root extension into the substratum at our grass bed sites.

Gill ultrastructure and histochemical staining

Both transmission electron microscopy of gill tissue and scanning electron microscopy of freeze-fractured gills reveal densely packed assemblages of rod-shaped procaryotic inclusions (Fig. 1A–C). The bacteria range from 4 to 6 microns in length (1–1.5 microns in diameter) and appear to be enclosed within vacuoles; the scanning electron micrograph seen in Figure 1A illustrates the high density of bacteria within the tissue as well as empty vacuolar spaces where bacteria have apparently fallen out during the freeze-fracturing process. In the higher magnification micrograph (Fig. 1B) bacteria residing within vacuoles are also visible just beneath the freeze-fracture plane. Figure 1C is a transmission electron micrograph of these inclusions showing their procaryotic-like structures (*i.e.*, no membrane-bound organelles and a distinct nuclear zone) and the vacuolar membrane that surrounds them.

The distribution of bacteria within the gill is not random, but rather, is restricted to a particular cell type. Figure 2A shows the ciliated gill epithelium to be a simple columnar epithelium composed of cells with large nuclei and an abundance of smaller inclusions, which at higher magnification are identifiable as mitochondria (Fig. 2A, inset). Bacteria have not been observed in this outer cellular layer. However, underlying the gill epithelium is a tissue composed of very large cells (up to 60 microns in diameter) which are rich in bacteria-containing vacuoles (Fig. 2B). In fact, the bacteria are easily the most abundant inclusion in the ground substance of the bacteriocyte. Autophagic lysosomes containing myelin-like figures and small vesicles are occasionally

TABLE I

Hydrogen sulfide concentrations in the interstitial water of sediments and population densities of L. floridana *in* Thalassia *and* Ruppia *seagrass beds*

Seagrass beds	Hydrogen sulfide (m*M*)	Population density of *L. floridana* (per m^2)
Thalassia	1.67 ± 0.31 (n = 13)	84 ± 12 (n = 21)
		83 ± 11 (average for collection site; n = 33)
Ruppia	2.49 ± 0.55 (n = 13)	74 ± 34 (n = 9)

An overall average for *Lucina* density is also presented which reflects the relative contribution from each seagrass type at the collection site. Values represent means plus or minus 95% confidence limits.

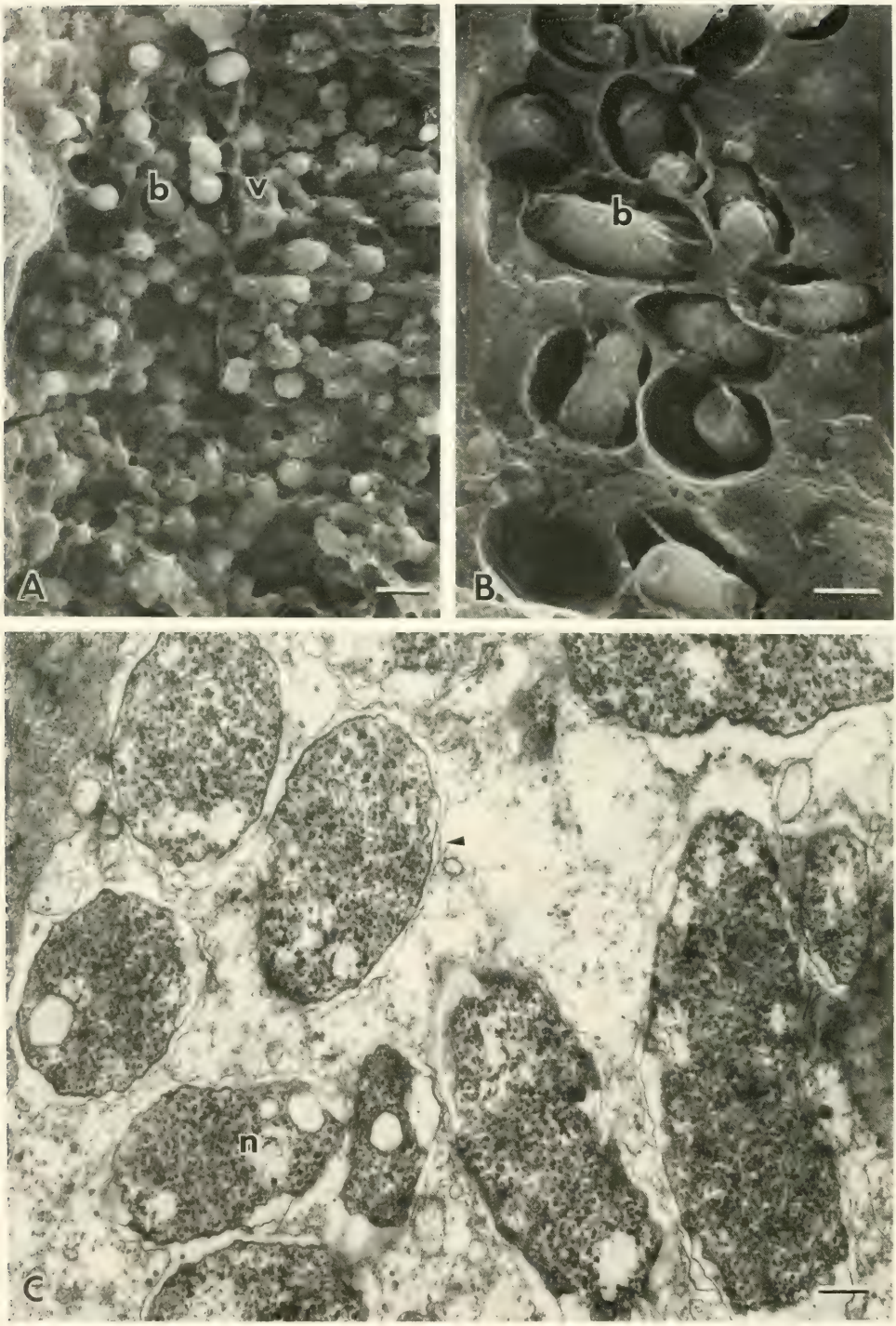

FIGURE 1. A. Scanning electron micrograph of freeze-fractured gill tissue, showing numerous bacteria (b) and empty vacuoles (v). Scale bar = 5 μm. A higher magnification is illustrated in B, where bacteria are also visible beneath the freeze-fracture plane. Scale bar = 2 μm. C. Transmission electron micrograph of the endosymbionts, which demonstrates their procaryotic structure, as noted by the nuclear region (n) and the lack of membrane-bound organelles. Arrow indicates vacuolar membranes surrounding a bacterium. Rarely is there more than one bacterium per vacuole. Scale bar = 0.5 μm.

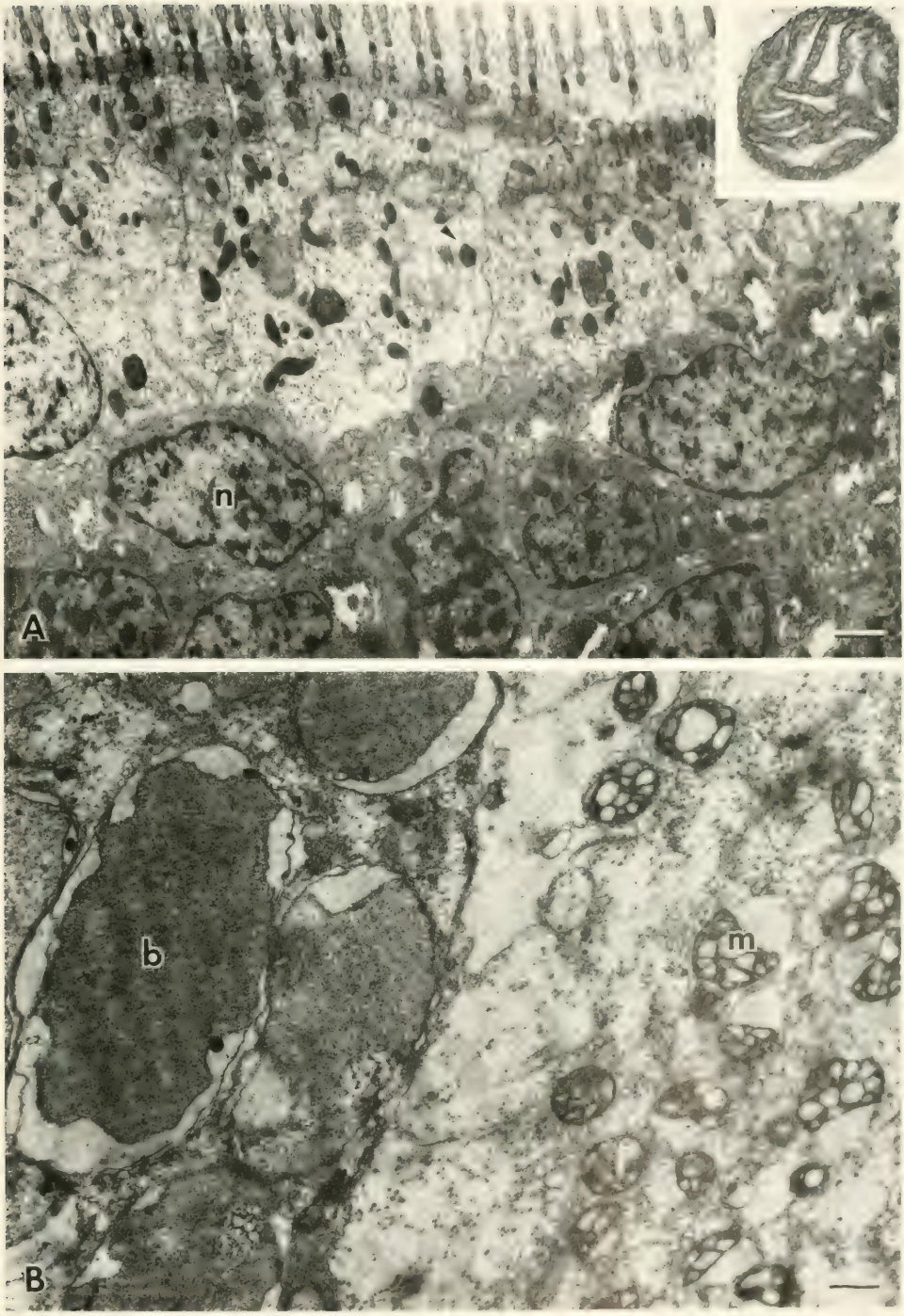

FIGURE 2. A. Transmission electron micrograph of a thin section through the gill epithelium, which is identifiable by its ciliated surface. Several large nuclei (n) are visible. Scale bar = 1 μm. The small inclusions (arrow) are mitochondria, as shown by the higher magnification in the inset. The lower micrograph, B, represents an interface between a bacterial-containing cell (bacteriocyte) and a cell possessing abundant mitochondria (m) but no bacteria. Scale bar = 0.5 μm.

observed, while mitochondria are rare. Bacteriocyte nuclei are generally quite small, having diameters of approximately 5 microns.

Also associated with the bacteriocytes are numerous intracellular pigment granules, 6–14 microns in diameter; we have not observed any such granules within the ciliated epithelial cells of the gill. Each bacteriocyte, however, may contain from one to several of these granules (Fig. 3A), which are surrounded by numerous bacterial endosymbionts (Fig. 3B). When thick sections of unstained gill are viewed with light-level microscopy, the pigment granules have a distinct yellow-brown coloration. Staining these sections for occult iron causes the granules to turn green, which is a positive result for the presence of iron.

Enzymatic analyses

Activities of ribulose 1,5-bisphosphate carboxylase (RuBPCase), a CO_2-fixing enzyme diagnostic of the Calvin-Benson cycle, were found in the gill tissue of *L. floridana* at levels comparable to those reported in fresh spinach leaves (Wishnick and Lane, 1971). Catalytic activity increased linearly with increasing volumes of homogenate, indicating enzyme proportionality (Fig. 4). From the substrate saturation curve determined for ribulose 1,5-bisphosphate, the apparent K_m was estimated to be 0.38 m*M* and the V_{max} 3.4 units/g wet weight tissue. This substrate becomes inhibitory at concentrations exceeding approximately 1.2 m*M*, consistent with substrate inhibition values for RuBPCase from other sources (Wishnick and Lane, 1971). Omitting the activity values measured above 1.2 m*M* ribulose, 1,5-bisphosphate, double reciprocal plots yielded quite different values of 4.25 m*M* and 18.3 units/g tissue for the apparent

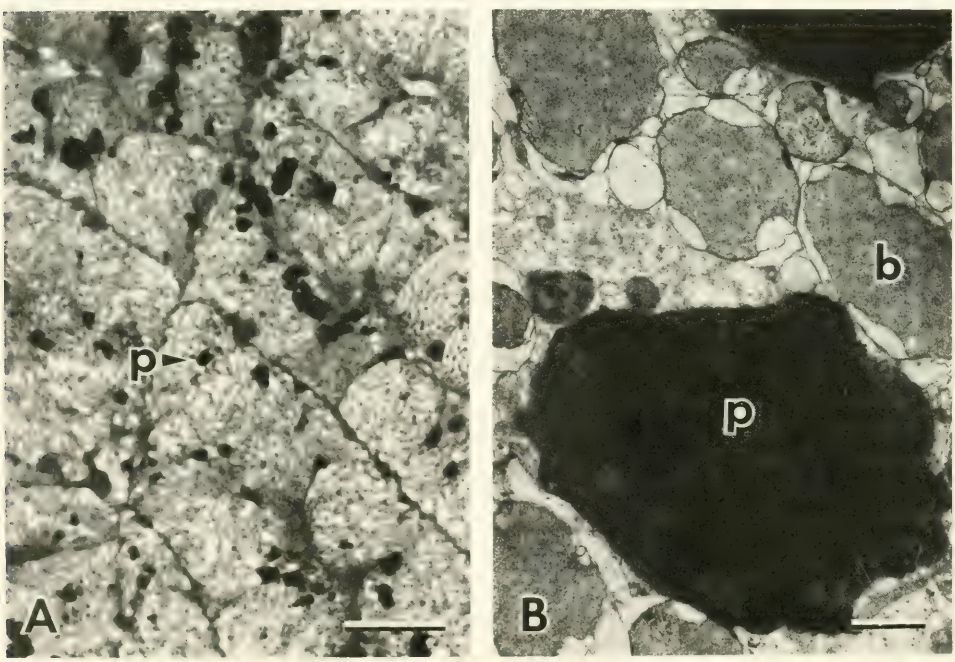

FIGURE 3. A. Light-level micrograph demonstrating the high numbers of pigment granules (p) within the bacteriocytes of the demibranch. Scale bar = 50 μm. B. Higher magnification shows the electron-dense pigment granules in close proximity to the bacteria (b). Scale bar = 1 μm.

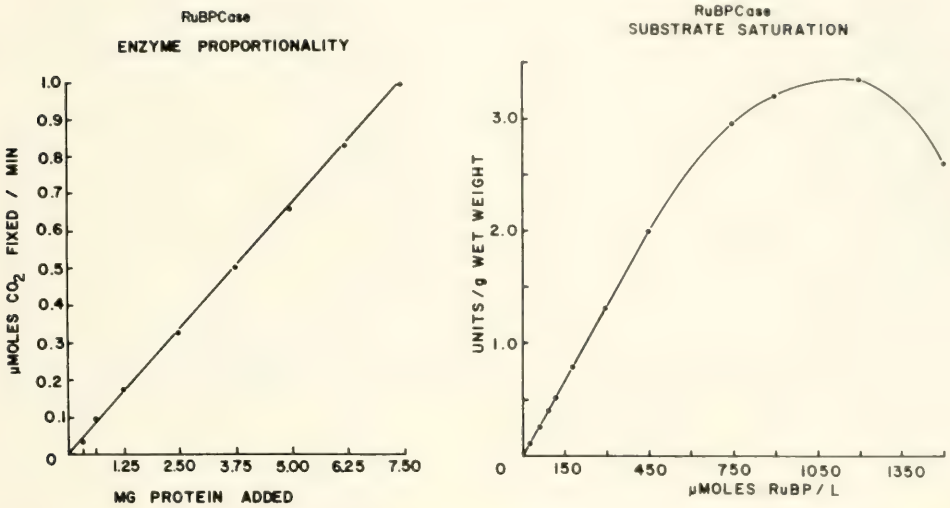

FIGURE 4. Enzyme proportionality and substrate saturation curves of ribulose 1,5-bisphosphate carboxylase (RuBPCase). Ribulose 1,5-bisphosphate becomes inhibitory at concentrations exceeding 1.2 m*M*, resulting in an approximate K_m and V_{max} of 0.38 m*M* and 3.4 units/g, respectively for the uninhibited portion of the curve.

K_m and V_{max}. Phosphoribulokinase, a second enzyme of the Calvin-Benson cycle, catalyzes the phosphorylation of ribulose-5-phosphate to ribulose 1,5-bisphosphate, and activity of this enzyme was also present in gill homogenates (Fig. 6). The activity was proportional to the amount of homogenate added, and a double reciprocal plot yielded a K_m of 0.38 m*M* for ribulose-5-phosphate and a V_{max} of 0.93 units/g wet weight. The presence of these two enzymic activities is consistent with a potential for net CO_2 fixation *in vivo* in the gill tissue.

Results of our enzymatic analyses for APS reductase and ATP sulfurylase are given in Figure 5. APS reductase catalyzes the production of adenosine phosphosulfate from AMP and sulfite, and a double reciprocal plot gave a V_{max} of 2.1 units/g and a K_m value of 0.26 μM for sulfite. ATP sulfurylase phosphorylates adenosine phosphosulfate to form ATP and sulfate; V_{max} and K_m values were 2.9 units/g wet weight gill tissue and 0.13 μM, respectively. Strong enzyme proportionality was observed for both activities. Although the enzyme rhodanese is not as reliable an indicator of sulfur-based energy metabolism as the two enzymes above, it is noteworthy that we were able to qualitatively demonstrate the presence of this enzymic activity in gill homogenates also. However, since the assay technique did not give linear reaction rates we cannot report a quantitative value.

Finally, nitrite reductase, which catalyzes the ferredoxin-dependent formation of ammonia from nitrite, was also measured in *Lucina* gill tissue at a level of 9.4 units/g (V_{max}). The K_m value for nitrite was determined to be 4.0 μM (Fig. 6).

Energy dispersive X-ray analysis

Sulfur was shown to be the dominant inorganic element in air-dried gill tissue (Fig. 7). Attempts to localize the sulfur in specific regions of the tissue by dot mapping suggested that the element was uniformly distributed; using this technique there was no evidence for the existence of concentrated sulfur deposits within the yellowish-

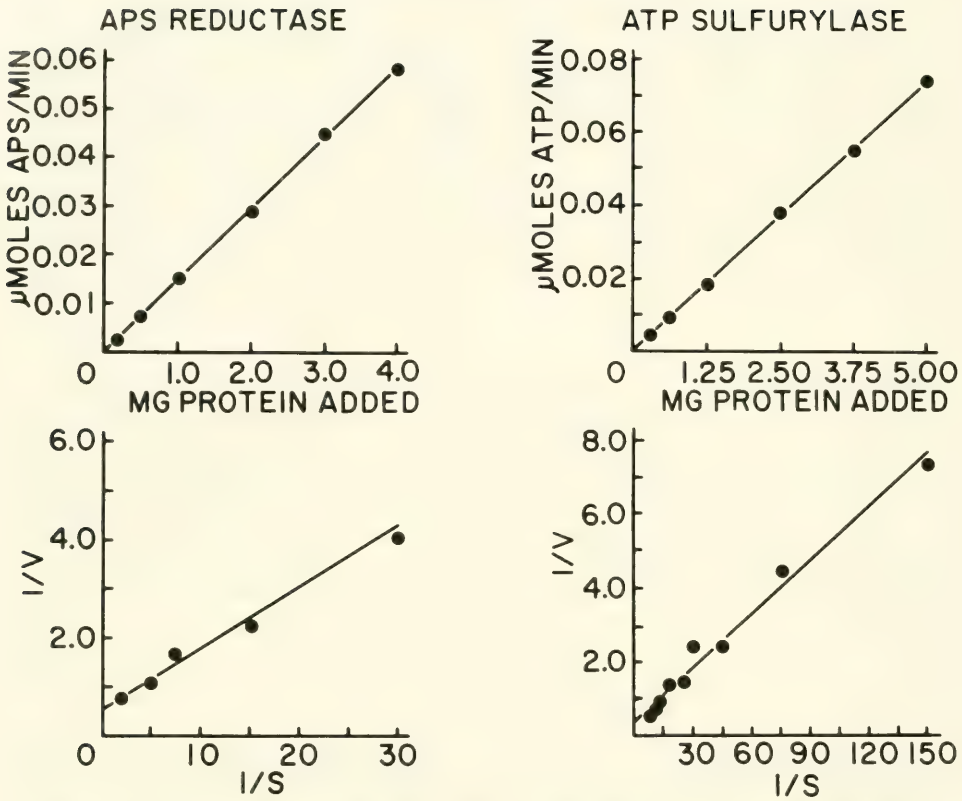

FIGURE 5. Enzyme proportionality and double reciprocal plots of APS reductase (left) and ATP sulfurylase (right). Both enzymes exhibit proportional activities, which increase linearly with the amount of added homogenate. Double reciprocal plots give V_{max} and apparent K_m values of 2.1 units/g and 0.26 μM for APS reductase and 2.9 units/g and 0.13 μM for ATP sulfurylase.

brown pigment granules. Leaching of sulfur from the tissue, as indicated by reduction in size of the sulfur peak, occurred if the samples were processed with an acetone dehydration series and critical point drying. The aluminum peak seen in the elemental spectral scan is an artifact due to the aluminum stub on which specimens were mounted.

DISCUSSION

The above results support the existence of bacterial endosymbionts in the gills of *Lucina floridana.* Confinement of these bacteria to vacuoles within the eucaryotic cells (Fig. 1) is the same morphological arrangement recently reported by Cavanaugh (1983) for gram-negative, symbiotic bacteria in the marine bivalves *Calyptogena magnifica, Solemya velum,* and *Lucinoma annulata* and by Felbeck (1983) for *Solemya reidi.* With the exception of rhodanese (which can also serve as a detoxifying enzyme in certain eucaryotes; Sorbo, 1953), the enzymes that we have demonstrated in the gill tissue of *L. floridana* are normally characteristic of chemoautotrophic sulfur bacteria. RuBPCase and ATP sulfurylase activities have been measured in the trophosome tissue of the vestimentiferan tube worm *Riftia pachyptila* by Felbeck (1981),

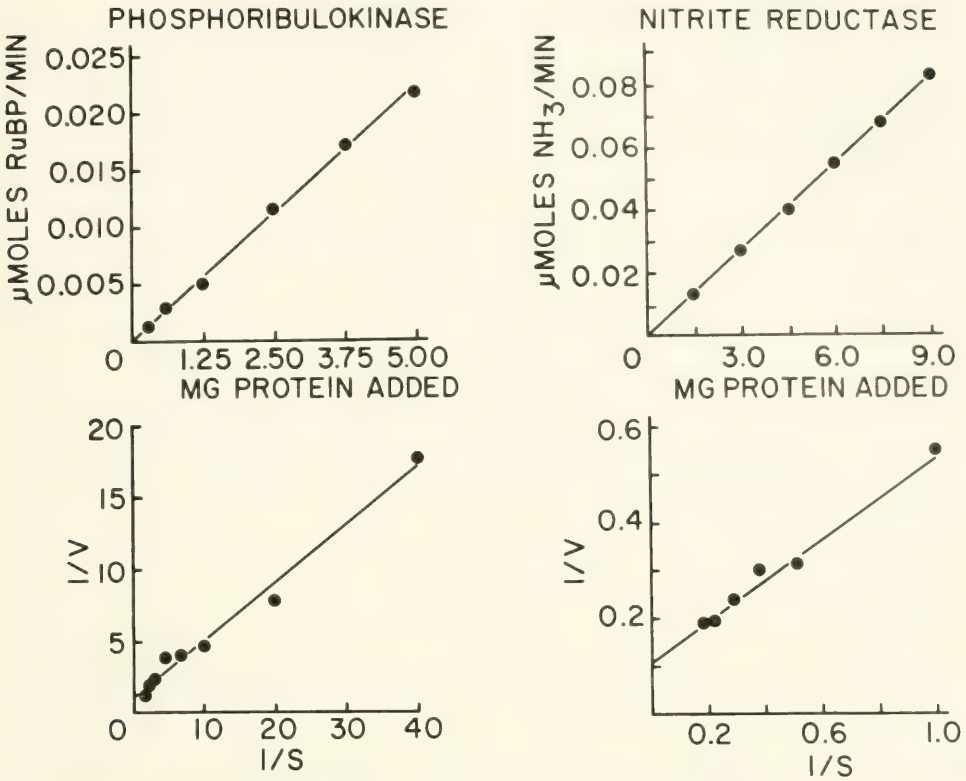

FIGURE 6. Enzyme proportionality and double reciprocal plots of phosphoribulokinase (left) and nitrite reductase (right). The calculated V_{max} and apparent K_m were 0.93 units/g and 0.38 μM for phosphoribulokinase and 9.4 units/g and 4.0 μM for nitrite reductase. Again, excellent enzyme proportionality was observed in both cases.

the pogonophoran *Lamellibrachia barhami* (Felbeck *et al.*, 1981), marine oligochaetes of the genus *Phallodrilus* (Felbeck *et al.*, 1983b), and the bivalves *Calyptogena pacifica, Lucinoma annulata, Parvilucina tenuisculpta, Solemya panamensis,* and *S. reidi* (Felbeck *et al.*, 1981; Felbeck, 1983). For several of these animal-bacterial symbioses, the enzymes phosphoribulokinase and APS reductase also have been documented (for review, see Felbeck *et al.*, 1983a). In the present study with *L. floridana* RuBPCase levels (3.4 units per gram fresh weight of tissue) are the highest reported thus far, with the activities for the other three enzymes falling well within the range of currently-existing values for similar symbioses (Felbeck *et al.*, 1983a). We also report here the presence of nitrite reductase, which has not been noted in any of the other studies above. Thus, it is likely that these bacterial endosymbionts provide *L. floridana* with the potential for chemoautotrophic metabolism fueled by sulfur oxidation. To substantiate this relationship, however, one should be able to demonstrate a sulfide-dependent synthesis of ATP and NADPH in the bacteriocyte, as well as the subsequent release of reduced carbon compounds from the bacteriocytes and transport to other tissues of the host.

For significant sulfide oxidation to occur within the tissue of *L. floridana* the animal must have access to both HS^- and O_2 in its immediate environment. The

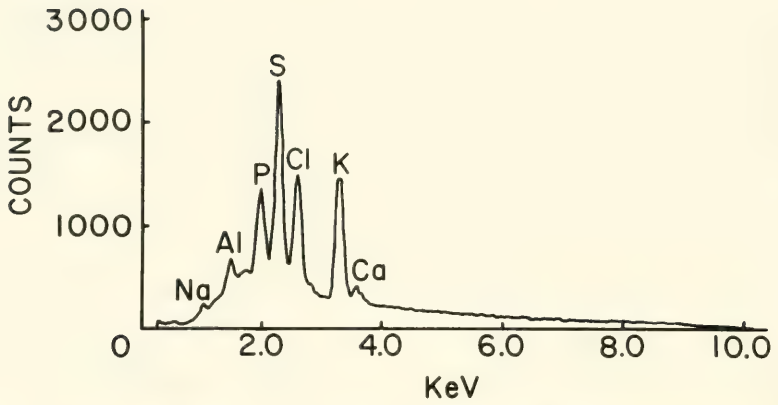

FIGURE 7. Energy dispersive X-ray analysis of air-dried, freeze-fractured *Lucina* gills. Period of analysis is 100 seconds. Sulfur (K_α = 2.3 KeV, K_β = 2.5 KeV) is clearly the dominant inorganic element, while Fe (K_α = 6.4 KeV, K_β = 7.1 KeV) is not detectable. The Al peak is an artifact due to the aluminum stub used for mounting the specimen.

abundance of sulfide is evident from our analyses of core samples taken from the habitat (Table I), and we have observed that the bivalve typically resides in close association with the O_2-releasing root systems of the seagrasses in these sediments. The size of oxygen-containing zones around the roots, in what is otherwise anoxic sediment, is dependent on the magnitude of O_2 flux from the roots and the porosity of the sediment, both of which can be quite variable (Armstrong, 1970). While these oxidized rhizospheres may serve to detoxify potential phytotoxins (*e.g.*, HS^-) as they come into contact with these zones (Armstrong, 1970), it seems reasonable that such rhizospheres could also serve as an O_2 source for *L. floridana;* we have not observed inhalant siphon tubes constructed by the bivalve of sufficient length to allow access to the oxygen in the overlying waters. Thus, while the actual oxygen concentration to which *Lucina* might be exposed has not been measured, it is clear that the animal's location in the sediment could place it at an oxygen/sulfide interface.

As is the case with other lucinids, *L. floridana* appears to have a functional gut. However, Allen (1958) described the digestive system of lucinids as being simplified, with reduction of palps, loss of sorting area, and reduction in the number of restrictions between the stomach and digestive diverticula. Allen suggested that these reductions/alterations of the gut were to facilitate the acceptance of larger food particles like detritus which is common in *Thalassia* beds (Fenchel, 1977); we have indeed noted small amounts of detritus in the gut of freshly-collected specimens. Allen further emphasized that the loss of sorting mechanisms would be expected in an environment where food supply was low and all available particulates must be accepted. Thus, the selective advantage to *L. floridana* of a chemoautotrophic potential in such a habitat would seem clear.

The presence of iron, localized in the pigment granules of *L. floridana* gills using histochemical staining, suggests that respiratory pigments (*e.g.*, myoglobin, hemoglobin) and/or even iron-containing cytochromes could be components of these electron-dense structures. Iron was not detected by energy dispersive X-ray analysis; neither the K_α peak at 6.4 KeV nor the K_β peak at 7.1 KeV was resolvable. However, this observation would not be unexpected if the iron present in these granules was indeed complexed with specific proteins. Under this condition, the iron concentration would be extremely low and possibly below the detection limit of the instrument. Sulfur,

on the other hand, was readily detected by X-ray analysis and was shown to be the dominant inorganic element in air-dried gill tissue. We were unable to specifically localize deposits of this element in the tissue using dot mapping. While structures like the pigment granules should have been within the resolving power of the technique, elemental sulfur deposited within vacuoles of the symbiotic bacteria (as is commonly observed in the Thiobacilli; Trudinger, 1969) would not have been localized due to their small size.

The occurrence of these pigment granules in the gills of lucinid clams has been known for some time. Allen (1958) noted the presence of "brown pigment granules" in the gill tissue of lucinids but offered no possible function. Read (1962) reported that the granules found in the "dark purplish" gills of *Phacoides pectinatus,* another lucinid, contained iron and identified the pigment granules as hemoglobin based on data obtained from absorption spectra and oxygen-combining properties of whole gill homogenates. Jackson (1973) noted the unusual dark coloration (presumably due to the presence of similar pigment granules) of the gills of the lucinids *Parvilucina costata, Lucina pennsylvanica, Anadontia alba, Codakia orbicularis,* and *Ctena orbiculata* and also concluded that the gills contained hemoglobin. Neither author, however, demonstrated clearly that the pigment granules were the source of the hemoglobin. We are currently isolating both bacteriocytes and pigment granules using cell dissociation and density gradient centrifugation in order to approach this question, as well as other ones regarding the proposed sulfide-driven, chemoautotrophic metabolism in this tissue.

One very interesting aspect regarding the morphological placement of the bacteriocytes within the eulamellibranch gill of *L. floridana* is that these cells are restricted to the interior of the organ and are not found in the ciliated epithelium (Fig. 2). The cells composing the epithelium are devoid of bacteria, yet have high densities of mitochondria. Bacteriocytes have a similar locus in the gill of the protobranch bivalve *Solemya* (Cavanaugh, 1983; Felbeck, 1983), such that again the bacterial-containing cells are shielded from the external environment by mitochondria-rich cells (or at least by cytoplasmic extensions of these cells). Since lucinids are known to be stenohaline osmoconformers (Jackson, 1973), an epithelium rich in mitochondria presumably would not be required for active, transepithelial movement of ions. Thus, we propose that one possible reason for such a spatial arrangement is that the gill epithelium may perform an oxygen-scavaging role, protecting oxygen-sensitive enzymes found in the deeper bacteriocytes from high O_2 concentrations. An analogy can be drawn to the leaf morphology of C_4 plants, where the Calvin-Benson cycle is localized in bundle-sheath cells surrounded by an outer layer of mesophyll cells. Since RuBPCase can exhibit both oxygenase and carboxylase activities due to competition between O_2 and CO_2 for the same active site, CO_2 fixation proceeds most efficiently when the intracellular ratio of CO_2 to O_2 is high, allowing normal hexose assimilation (Chollet, 1977). An opposite ratio promotes glycolate formation and the wasteful process of photorespiration. Thus, the high CO_2:O_2 ratio maintained in bundle-sheath cells is functionally beneficial to C_4 plants, and the location of bacteriocytes within *L. floridana* gill tissue could foster efficient functioning of bacterial RuBPCase for similar reasons. Reduced O_2 diffusion to the bacteriocytes might also be beneficial to other bacterial, oxygen-sensitive enzymes like nitrate reductase (Stouthamer, 1976) and sulfur oxidase (Kelly, 1982). Furthermore, if work with isolated pigment granules reveals the presence of oxygen-binding proteins, then these structures could serve as a source of sequestered oxygen to be delivered to bacteriocytes for processes like sulfur oxidation. It should be noted that a high-affinity hemoglobin has recently been documented as an intracellular component of *Solemya velum* gills (Doeller *et al.,* 1983).

Based on average values for population density, grams of fresh gill tissue per individual (0.42 ± 0.07; 95% CI, n = 8), and half-maximal velocities of RuBPCase as determined *in vitro* (1.51 ± 0.26 units/g tissue; 95% CI, n = 8), *L. floridana* could potentially contribute 336 ± 96 g C/m^2/year (95% CI) to the gross carbon fixation of the seagrass beds, a component of gross productivity that has not been considered up to now. Of course, this fixation value is only an estimate, since *in vitro* enzyme activity values are at best only reflective of *in situ* metabolic potential. The contribution to net carbon fixation of seagrass ecosystems, if any, can only be assessed after completion of energy budget studies designed to answer how reduced carbon is partitioned among growth, storage, and routine metabolism in *L. floridana.*

ACKNOWLEDGMENTS

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LITERATURE CITED

ALLEN, J. A. 1958. On the basic form and adaptations to habitat in the Lucinacea (Eulamellibranchia). *Phil. Trans. R. Soc. Lond. B* **241**: 421–484.

ARMSTRONG, W. 1970. Rhizosphere oxidation in rice and other species: a mathematical model based on the oxygen flux component. *Physiol. Plant.* **23**: 623–630.

BAUMANN, E. W. 1974. Determination of parts per billion sulphide in water with the sulfide-selective electrode. *Anal. Chem.* **46**: 1345–1347.

BERG, C. J., JR., AND P. ALATALO. 1984. Potential of chemosynthesis in molluscan mariculture. *Aquaculture* **39**; 165–179.

BRITTON, J. C., JR. 1970. The Lucinidae (Mollusca: Bivalvia) of the western Atlantic Ocean. Ph.D. Dissertation, The George Washington University, District of Columbia.

CAVANAUGH, C. M. 1983. Symbiotic chemoautotrophic bacteria in marine invertebrates from sulphide-rich habitats. *Nature* **302**: 58–61.

CAVANAUGH, C. M., S. GARDINER, M. K. JONES, H. W. JANNASCH, AND J. B. WATERBURY. 1981. Prokaryotic cells in the hydrothermal vent tube worm *Riftia pachyptila* Jones: possible chemoautotrophic symbionts. *Science* **213**: 340–342.

CHOLLET, R. 1977. The biochemistry of photorespiration. *Trends Biochem. Sci.* **2**: 155–159.

DOELLER, J. E., D. W. KRAUS, AND J. M. COLACINO. 1983. The presence of hemoglobin in *Solemya velum* (Bivalvia, Protobranchia). *Am. Zool.* **23**(4): 976.

EDMOND, J. M., K. L. VON DAAM, R. E. MCDUFF, AND C. I. MEASURES. 1982. Chemistry of hot springs on the East Pacific Rise and their effluent dispersal. *Nature* **297**: 187–191.

EDWARDS, P. 1976. *Illustrated Guide to the Seaweeds and Sea Grasses in the Vicinity of Port Aransas, Texas.* University of Texas Press, Austin. 130 pp.

FELBECK, H. 1981. Chemoautotrophic potential of the hydrothermal vent tube worm, *Riftia pachyptila* Jones (Vestimentifera). *Science* **213**: 336–338.

FELBECK, H. 1983. Sulfide oxidation and carbon fixation by the gutless clam *Solemya reidi:* an animal-bacteria symbiosis. *J. Comp. Physiol.* **152**: 3–11.

FELBECK, H., AND G. N. SOMERO. 1982. Primary production in deep-sea hydrothermal vent organisms: roles of sulfide-oxidizing bacteria. *Trends Biochem. Sci.* **7**: 201–204.

FELBECK, H., J. J. CHILDRESS, AND G. N. SOMERO. 1981. Calvin-Benson cycle and sulphide oxidation enzymes in animals from sulphide-rich habitats. *Nature* **293**: 291–293.

FELBECK, H., J. J. CHILDRESS, AND G. N. SOMERO. 1983a. Biochemical interactions between molluscs and their algal and bacterial symbionts. Pp. 331–358 in *The Mollusca,* Vol. 2., P. W. Hochachka, ed. Academic Press, New York.

FELBECK, H., G. LIEBEZEIT, R. DAWSON, AND O. GIERE. 1983b. CO_2 fixation in tissues of marine oligochaetes (*Phallodrilus leukodermatus* and *P. planus*) containing symbiotic, chemoautotrophic bacteria. *Mar. Biol.* **75**: 187–191.

FENCHEL, T. 1977. Aspects of the decomposition of seagrasses. Pp. 123–145 in *Seagrass Ecosystems: A Scientific Perspective,* C. P. McRoy and C. Helfferich, eds. Marcel Dekker, Inc., New York.

FENCHEL, T., AND R. J. RIEDL. 1970. The sulfide system: a new biotic community underneath the oxidized layer of marine sand bottoms. *Mar. Biol.* **7:** 255–268.

JACKSON, J. B. C. 1973. The ecology of the molluscs of *Thalassia* communities, Jamaica, West Indies. I. Distribution, environmental physiology and ecology of common shallow water species. *Bull. Mar. Sci.* **23**(2): 313–347.

JORGENSEN, B. B., AND T. FENCHEL. 1974. The sulfur cycle of a marine sediment model system. *Mar. Biol.* **24:** 189–201.

KELLY, D. P. 1982. Biochemistry of the chemolithotrophic oxidation of inorganic sulfur. *Phil. Trans. R. Soc. Lond. B.* **298:** 499–528.

KIKUCHI, T., AND J. M. PERES. 1977. Consumer ecology of seagrass beds. Pp. 107–134 in *Seagrass Ecosystems: A Scientific Perspective,* C. P. McRoy and C. Helfferich, eds. Marcel Dekker, Inc., New York.

LOSADA, M., AND A. PANEQUE. 1971. Nitrite reductase. *Methods Enzymol.* **23**A: 487–491.

MILLONIG, G. 1976. *Laboratory Manual of Biological Electron Microscopy.* Privately printed, Italy. 67 pp.

PECK, H. D., T. E. DEACON, AND J. T. DAVIDSON. 1965. Studies on adenosine 5′-phosphosulfate reductase from *Desulfovibrio desulfuricans* and *Thiobacillus thioparus.* I. The assay and purification. *Biochem. Biophys. Acta* **96:** 429–432.

PETERSON, G. 1977. A simplification of the protein assay method of Lowry *et al.* which is more generally applicable. *Anal. Biochem.* **83:** 345–356.

RACKER, E. 1957. The reductive pentose phosphate cycle. I. Phosphoribulokinase and ribulose diphosphate carboxylase. *Arch. Biochem. Biophys.* **69:** 300–305.

READ, K. R. H. 1962. The hemoglobin of the bivalved mollusc *Phacoides pectinatus* Gmelin. *Biol. Bull.* **123:** 605–617.

SMITH, A. J., AND J. LASCELLES. 1966. Thiosulphate metabolism and rhodanese in *Chromatium* sp. strain D. *J. Gen. Microbiol.* **42:** 357–370.

SORBO, B. 1953. Crystalline rhodanese. I. Purification and physicochemical examination. *Acta Chem. Scand.* **7:** 1129–1136.

STOUTHAMER, A. H. 1976. Biochemistry and genetics of nitrate reductase in bacteria. *Adv. Microbial Physiol.* **14:** 315–370.

TEAL, J. M., AND J. W. KANWISHER. 1966. Gas transport in the marsh grass, *Spartina alterniflora. J. Exp. Bot.* **17:** 355–361.

TRUDINGER, P. A. 1969. Assimilatory and dissimilatory metabolism of inorganic sulfur compounds by microorganisms. *Adv. Microbial Physiol.* **3:** 111–152.

WISHNICK, M., AND D. LANE. 1971. Ribulose diphosphate carboxylase from spinach leaves. *Methods Enzymol.* **23:** 570–577.

ZIEMAN, J. C., AND R. G. WETZEL. 1980. Productivity in seagrasses: methods and rates. Pp. 115–131 in *Handbook of Seagrass Biology: An Ecosystem Perspective,* R. C. Phillips and C. P. McRoy, eds. Garland STPM Press, New York.

Reference: *Biol. Bull.* **167**: 460–466. (October, 1984)

THE FMRFamide-LIKE NEUROPEPTIDE OF *APLYSIA* IS FMRFamide

H. K. LEHMAN*, D. A. PRICE, AND M. J. GREENBERG

C. V. Whitney Laboratory, University of Florida, Route 1, Box 121, St. Augustine, Florida 32086

ABSTRACT

The head ganglia from 350 *Aplysia brasiliana* were extracted and purified by gel (Sephadex G-15) and cation exchange (CM-Sephadex) chromatography; the fractions were examined with radioimmunoassays (RIA) for the molluscan neuropeptides, FMRFamide and SCP_B. Immunoreactive (ir-) FMRFamide (but not ir-SCP_B) coeluted with authentic FMRFamide from both chromatographic columns. The amino acid composition of the purified peptide was: Phe 2: Arg 1: Met 1. Digestion of purified ir-FMRFamide with carboxypeptidase Y indicated that the four residues were in the same sequence as occurs in FMRFamide. The dose-response curves for purified and synthetic FMRFamide on the radula protractor muscle of *Busycon contrarium* were coincident, as were their inhibition binding curves in the FMRFamide RIA. The highest concentrations of ir-FMRFamide were in the pedal and pleural ganglia; but SCP_B was concentrated in the buccal ganglion. Synthetic SCP_B has no effect on the radula protractor muscle of *Busycon* or the isolated heart of *Mercenaria.* In conclusion, the FMRFamide-like peptide in the gastropod *Aplysia* is FMRFamide, so this peptide has now been identified in two molluscan classes. Moreover, the proposed structural relationship between FMRFamide and SCP_B is fortuitous, and these two peptides should have different physiological functions in *Aplysia.*

INTRODUCTION

The neuropeptide FMRFamide (Phe-Met-Arg-Phe-NH_2) was originally isolated and identified from the ganglia of the clam *Macrocallista nimbosa* (Price and Greenberg, 1977a). More recently, a "small cardioactive peptide," SCP_B (Met-Asn-Tyr-Leu-Ala-Phe-Pro-Arg-Met-NH_2), was isolated from the opisthobranch gastropod, *Aplysia* (Morris *et al.,* 1982). On the basis of the modest similarity in the sequences of SCP_B and FMRFamide, a new peptide family including both peptides was suggested; the proposed minimum structural requirement for biological activity was Phe-A-Arg-B-NH_2 (where A and B are hydrophobic amino acids). However, we have found SCP_B to be inactive on the *Busycon* radula protractor and on the *Mercenaria* heart, both classic FMRFamide bioassays (Price and Greenberg, 1980). Furthermore, whereas SCB_B did not react in a FMRFamide radioimmunoassay (RIA), immunoreactive FMRFamide was detected in *Aplysia* ganglia in our preliminary experiments; and FMRFamide does affect neurons, gill, and the anterior gizzard of *Aplysia* (Stone and Mayeri, 1981; Weiss *et al.,* 1982; Austin *et al.,* 1983). Therefore, we suspected that *Aplysia* should possess another peptide more similar to FMRFamide than SCP_B. We now report that FMRFamide itself occurs in *Aplysia,* that it is chromatographically distinct from SCP_B, and that its distribution among the central ganglia of *Aplysia* is markedly different from that of SCP_B.

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* To whom correspondence should be addressed.

Materials and Methods

Purification

The purification of Price and Greenberg (1977b) was used to isolate immunoreactive (ir-) FMRFamide from an aqueous acetone extract of head ganglia (pedal, pleural, and cerebral ganglia) from 350 *Aplysia brasiliana.* The dried extract was taken up in 2.0 ml of 0.1 *M* acetic acid and fractionated on a 2.5 × 43.2 cm Sephadex G-15 column pre-equilibrated with 0.1 *M* acetic acid, and eluted with the same. Fractions (300 drops; about 8 ml) were collected, and 2 μl aliquots of each were taken for radioimmunoassay of ir-FMRFamide and ir-SCP_B. Approximately 90% of the total immunoreactivity was recovered from Sephadex G-15.

The fraction from Sephadex G-15 that contained most of the ir-FMRFamide was diluted with distilled water to .05 *M*, its pH was adjusted to 5.5, and it was applied to a 0.5 × 25.0 cm CM-Sephadex cation exchange column. The column was then washed with 20 ml of 50 m*M* ammonium acetate, and the sample was eluted with a 40 ml linear gradient extending from 50 m*M* ammonium acetate (pH 6.0) to 500 m*M* ammonium acetate (pH 8.0). Fractions (1 ml) were collected, and 2 μl samples were assayed for ir-FMRFamide.

Amino acid composition and sequence

The ion exchange fractions containing the majority of ir-FMRFamide were tested for purity by amino acid determination. Aliquots (200 μl) from the peak ion exchange fractions were lyophilized and hydrolyzed in 6 *M* HCl for 24 h, and their amino acid compositions were determined with an automatic analyzer (Hitachi, Model 835).

The ion exchange purified peptide was then subjected to sequential digestion with carboxypeptidase Y (Sigma). An enzyme solution (10 μl of a 1 mg/ml solution, 0.05 *M* sodium phosphate, pH 7.0) was added to one nanomole of lyophilized peptide. Digestion was stopped after 5 and 30 minutes by dilution of the sample in .025 *N* HCl, and the amino acids liberated were determined by amino acid analysis.

Bioassays

Isolated *Busycon* radula protractor muscles were prepared by the method of Hill (1958) and suspended in an organ bath containing aerated, natural sea water. Tension was recorded with a force-displacement transducer connected to an inkwriting oscillograph. All drugs were added directly to the bath, and doses of FMRFamide are expressed as the final molar concentration in the bath.

The radioimmunoassays

Radioimmunoassays (RIA) were used to detect ir-FMRFamide and ir-SCP_B during the purification and isolation of FMRFamide. The FMRFamide RIA employed, as trace, iodinated Tyr-Gly-Gly-FMRFamide (YGGFMRFamide); the antiserum (S-253) is directed towards the COOH-terminal residues of FMRFamide. The assay is sensitive (IC_{50} = .3 pmoles) and specific to the FMRFamide molecule (Price, 1983). The SCP_B RIA employed iodinated SCP_B as the trace, and the antiserum was provided by H. R. Morris, Imperial College of London. The SCP_B assay is also sensitive (IC_{50} = .5 pmoles) and specific; its cross-reactivity with FMRFamide is only .01%, where SCP_B = 100%.

Each assay tube contained 100 μl of diluted antiserum, 50 μl of trace, and 50 μl of standard or unknown. Buffered saline (10 m*M* phosphate, containing 1% BSA,

0.01% merthiolate and 25 m*M* disodium EDTA; pH 7.6) was used to dilute the antiserum, trace, and standards. The tubes were incubated at 4°C for about 16 hours, and dextran-coated charcoal solution was then added to each tube to separate the bound and free antigen (1 ml of 2.5 g/l charcoal and 250 mg/l dextran in 10 m*M* phosphate buffer, pH 7.6).

Results

When the extract of *Aplysia* ganglia was applied to Sephadex G-15, ir-FMRFamide and ir-SCP_B were clearly separable (Fig. 1). Approximately 34.4 nmoles of ir-FMRFamide were recovered from fractions 20–23, whereas ir-SCP_B eluted earlier; 27.2 nmoles were recovered from fraction 15–18.

Fraction 22 from Sephadex G-15 contained the majority of ir-FMRFamide, and this material was retained on the cation exchange column. A single peak of ir-FMRFamide (6.3 nmoles) eluted from the ion exchanger in fractions 21, 22, and 23 (Fig. 2). Authentic FMRFamide behaves similarly on CM-Sephadex under these same experimental conditions (Price and Greenberg, 1977b; Greenberg and Price, 1979). On HPLC (μ-Bondapak C_{18} column), purified ir-FMRFamide and synthetic peptide coelute at the same position in a methyl alcohol: 0.1% trifluoroacetic acid gradient (30% methyl alcohol to 50% in 20 min); synthetic SCP_B elutes later.

Two amino acid analyses of the ion exchange purified peptide revealed the following compositions in nanomoles: Phe (.923; 1.194); Arg (.461; .566); and Met (.401; .408). The ratios are as expected for authentic FMRFamide (*i.e.*, about 2:1:1). Other amino

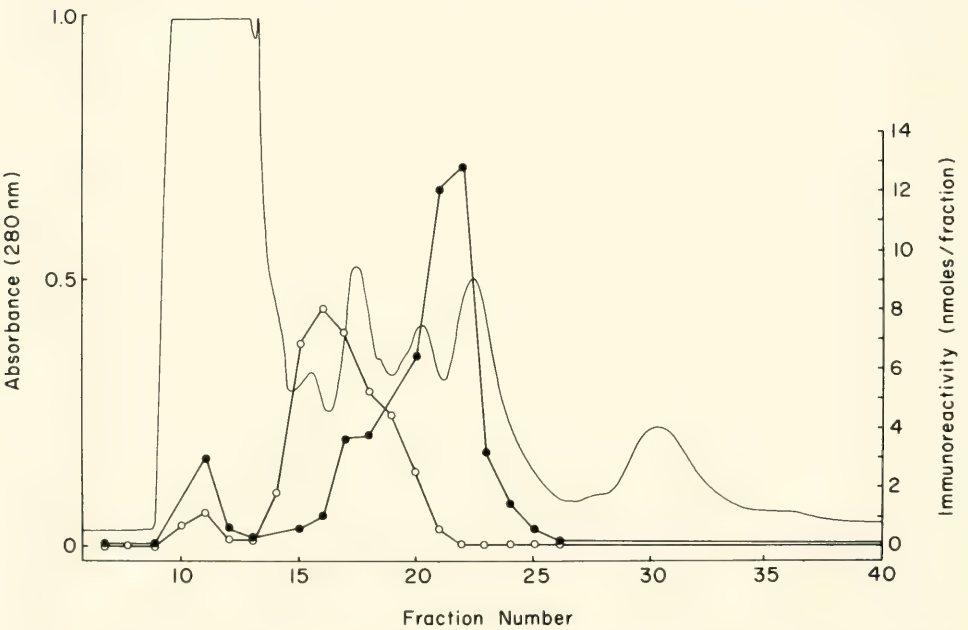

FIGURE 1. Separation of FMRFamide and SCP_B from *Aplysia* head ganglia by gel chromatography. An acetone extract of the head ganglia was passed through a 2.5 × 43.5 cm Sephadex G-15 column pre-equilibrated in 0.1 *M* acetic acid, and eluted with the same. Approximately 8 ml fractions (300 drops) were collected. Immunoreactive FMRFamide (solid circles) and SCP_B (open circles) were detected from 2 μl aliquots of each 8 ml fraction.

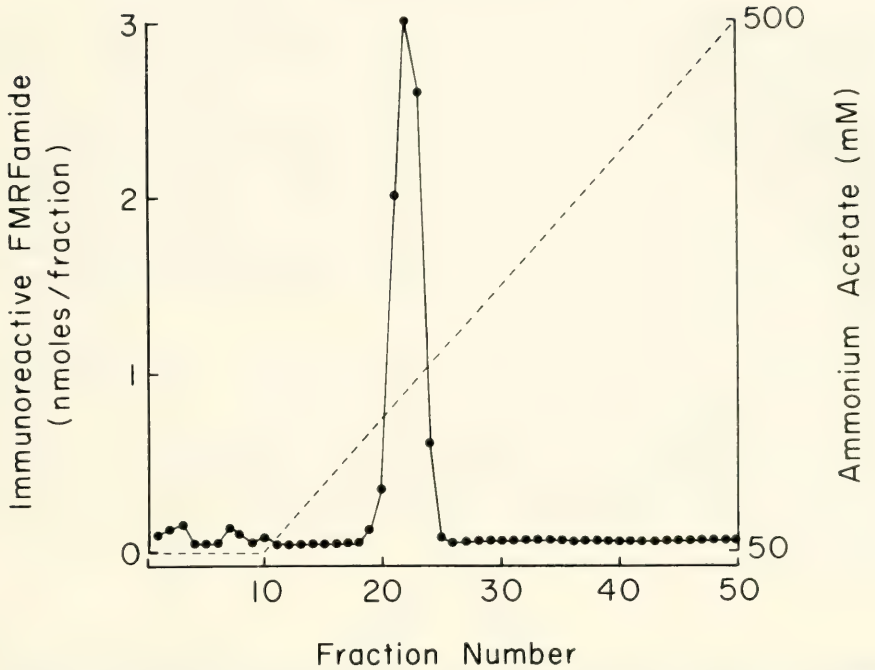

FIGURE 2. Purification of ir-FMRFamide by ion-exchange chromatography. Fraction 22 from Sephadex G-15 was applied to a CM-Sephadex cation exchange column and eluted with a 40 ml linear gradient, from 50 to 500 m*M* ammonium acetate. One ml fractions were collected, and 2 μl samples were assayed for immunoreactive FMRFamide.

acids detected included Gly (.109), Ser (.097), and Asp (.064), but they were considered to be contaminants.

Carboxypeptidase Y digestion yielded, after a 5 min digestion, only Phe (.705 nmoles) and Arg (.523 nmoles). The 30 min digestion released Phe (1.199 nmoles),

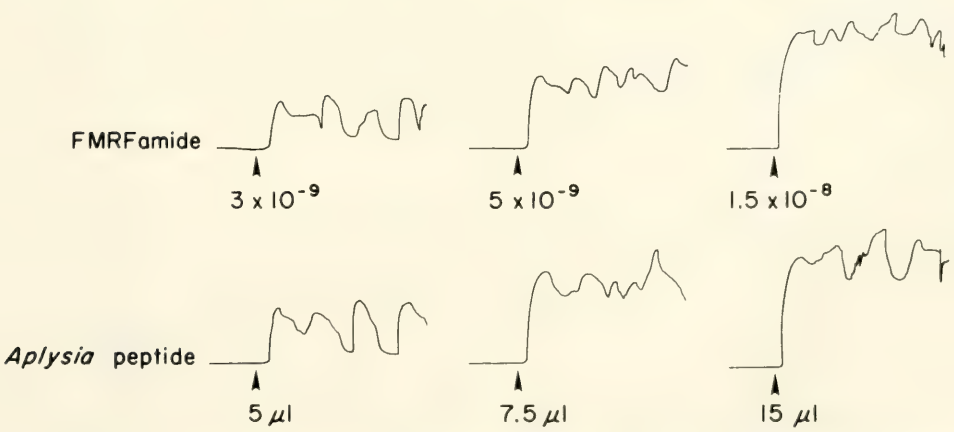

FIGURE 3. The mechanical responses of the *Busycon* radula protractor muscle to FMRFamide (upper recordings) and to aliquots of ion exchange fraction 23 (lower recordings).

Arg (.562 nmoles), and Met (.184 nmoles). Thus, the carboxypeptidase digestion indicated that the four residues were arranged in the same sequence occurring in FMRFamide.

The purified peptide from ion exchange fraction 23 was tested with the FMRFamide RIA and the *Busycon* radula protractor muscle. First, the effect of the peptide on the radula protractor muscle of *Busycon contrarium* was identical to that of FMRFamide (Fig. 3), and the dose response curves of both peptides were virtually coincident (Fig. 4). Synthetic SCP_B was also tested on the radula protractor muscle of *Busycon* and the isolated heart of the clam *Mercenaria.* It was inactive on these preparations at doses as high as 10^{-5} *M*. Second, the isolated ir-FMRFamide peptide produced an inhibition binding curve almost coincident to that of synthetic FMRFamide in the FMRFamide-specific RIA (Fig. 5).

In summary, the quantitative estimate of peptide content in ion exchange fraction 23, as determined by RIA (2.63 nmoles) and by bioassay (3.5 nmoles), closely agrees with that found by amino acid composition (3.2 nmoles).

The amount of ir-FMRFamide and ir-SCP_B in the pleural, pedal, buccal, cerebral, and abdominal ganglia were determined by RIA (Table I). Whereas the highest concentrations of ir-FMRFamide were in the pedal and pleural ganglia, SCP_B was concentrated primarily in the buccal ganglia, which contains the lowest levels of FMRFamide.

Discussion

We conclude that the FMRFamide-like peptide extracted and purified from *Aplysia* ganglia is authentic FMRFamide based on its amino acid composition, its sequence

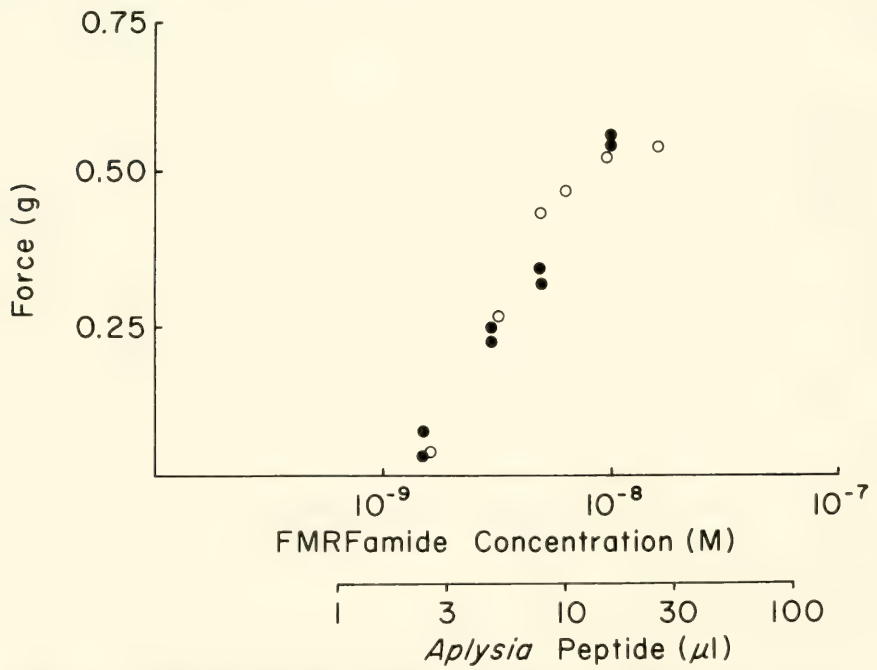

FIGURE 4. The log dose-response relations of FMRFamide (solid circles), and of aliquots of the purified *Aplysia* peptide in ion exchange fraction 23 (open circles), tested on the isolated radula protractor muscle of *Busycon contrarium.*

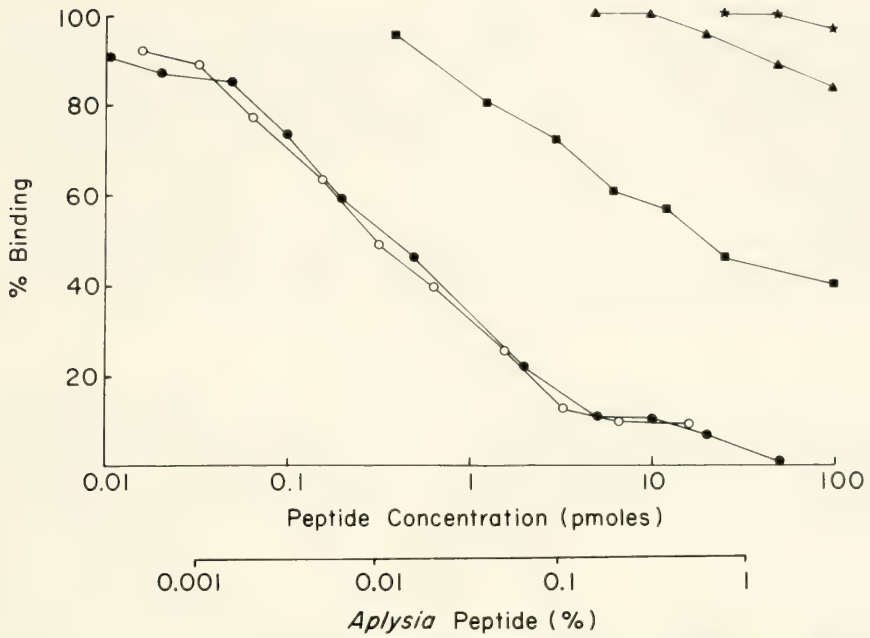

FIGURE 5. The displacement from antibody of ^{125}I-YGGFMRFamide, by FMRFamide FMRWamide, FMRLamide, YGGFMRF, and the purified *Aplysia* peptide. The dose of *Aplysia* peptide (lower abscissa) is expressed as a percentage of the 1 ml ion exchange fraction 23.

implied by carboxypeptidase Y digestion, and its immunological and biological activity.

We have now shown that the tetrapeptide Phe-Met-Arg-Phe-NH_2 exists in two widely separate molluscan classes, Gastropoda and Bivalvia (Price and Greenberg, 1977a). Furthermore, the pulmonate gastropod *Helix aspersa* contains an extended peptide with a very similar COOH-terminal tetrapeptide which is active in both immunological and biological assays for FMRFamide (Price, 1982). Therefore, the FMRFamide-like family of peptides in the phylum Mollusca is characterized by activity in specific definitive bioassays; and this activity, in turn, requires a COOH-terminal tetrapeptide of relatively invariant sequence (Painter *et al.*, 1982).

Immunoreactive FMRFamide and ir-SCP_B are not only chromatographically separable, they also have distinct distributions within the central ganglia of *Aplysia*. SCP_B

TABLE I

The distribution of ir-FMRFamide and ir-SCP_B in the central ganglia of Aplysia

	ir-FMRFamide	ir-SCP_B
Pedal	43.5 ± 10.7	23.1 ± 10.5
Pleural	34.3 ± 8.3	9.1 ± 3.0
Abdominal	18.6 ± 5.4	6.8 ± 3.6
Cerebral	15.7 ± 8.8	16.1 ± 8.7
Buccal	7.6 ± 2.5	75.0 ± 45.3

Values are given as pmoles/pair of ganglia ± standard deviation. Each value represents five separate determinations of five pairs of ganglia.

has only a slight structural resemblance to FMRFamide. In particular, its COOH-terminal tetrapeptide sequence, critical for FMRFamide-like activity, is markedly different; thus its lack of effect on both the radula protractor muscle and the clam heart was expected. Furthermore, several identified vertebrate peptides (*e.g.*, γ1 MSH and LPLRFamide) (Dockray *et al.*, 1983) are at least as similar to FMRFamide as is SCP_B; and SCP_B is as similar to bovine pancreatic polypeptide and arginine vasopressin as it is to FMRFamide.

In conclusion, the structural similarity between SCP_B and FMRFamide appears to be fortuitous; and the receptors, functions, and distribution of SCP_B in *Aplysia* should be distinct from those of FMRFamide. Our findings, that the distribution and activity of the two peptides are, in fact, different, support this conclusion.

ACKNOWLEDGMENTS

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LITERATURE CITED

AUSTIN, T., S. WEISS, AND K. LUKOWIAK. 1983. FMRFamide effects on spontaneous and induced contractions of the anterior gizzard in *Aplysia*. *Can. J. Physiol. Pharmacol.* **61**: 949–953.

DOCKRAY, G. J., J. R. REEVE, J. SHIVELY, R. J. GAYTON, AND C. S. BARNARD. 1983. A novel active pentapeptide from chicken brain identified by antibodies to FMRFamide. *Nature* **305**: 328–330.

GREENBERG, M. J., AND D. A. PRICE. 1979. FMRFamide, a cardioexcitatory neuropeptide of molluscs: an agent in search of a mission. *Am. Zool.* **19**: 163–174.

HILL, R. B. 1958. The effects of certain neurohumors and of other drugs on the ventricle and radula protractor of *Busycon canaliculatum* and on the ventricle of *Strombus gigas*. *Biol. Bull.* **115**: 471–482.

MORRIS, H. R., M. PANICO, A. KARPLUS, P. E. LLOYD, AND B. RINIKER. 1982. Elucidation by FAB-MS of the structure of a new cardioactive peptide from *Aplysia*. *Nature* **300**: 643–645.

PAINTER, S. D., J. S. MORLEY, AND D. A. PRICE. 1982. Structure-activity relations of the molluscan neuropeptide FMRFamide on some molluscan muscles. *Life Sci.* **31**: 2471–2478.

PRICE, D. A. 1982. The FMRFamide-like peptide of *Helix aspersa*. *Comp. Biochem. Physiol.* **72C**: 325–328.

PRICE, D. A. 1983. FMRFamide: assays and artifacts. Pp. 184–189 in *Molluscan Neuro-Endocrinology*, J. Lever and H. H. Boer, eds. Monogr. R. Neth. Acad. Arts Sci., North-Holland, Amsterdam.

PRICE, D. A., AND M. J. GREENBERG. 1977a. Structure of a molluscan cardioexcitatory neuropeptide. *Science* **197**: 670–671.

PRICE, D. A., AND M. J. GREENBERG. 1977b. Purification and characterization of a cardioexcitatory neuropeptide from the central ganglia of a bivalve mollusc. *Prep. Biochem.* **7**: 261–281.

PRICE, D. A., AND M. J. GREENBERG. 1980. Pharmacology of the molluscan cardioexcitatory neuropeptide FMRFamide. *Gen. Pharmacol.* **11**: 237–241.

STONE, L. S., AND E. MAYERI. 1981. Multiple actions of FMRFamide on identified neurons in the abdominal ganglion of *Aplysia*. *Soc. Neurosci. Abstr.* **7**: 636.

WEISS, S., J. GOLDBERG, G. I. DRUMMOND, AND K. LUKOWIAK. 1982. FMRFamide modulation of contractile behaviors of the isolated *Aplysia* gill. *Soc. Neurosci. Abstr.* **8**: 285.

Reference: *Biol. Bull.* **167**: 467–476. (October, 1984)

DIRECT ACCESS OF IONS TO THE SQUID STELLATE GANGLION GIANT SYNAPSE BY AORTIC PERFUSION: EFFECTS OF CALCIUM-FREE MEDIUM, LANTHANUM, AND CADMIUM

E. F. STANLEY* AND W. J. ADELMAN, JR.†

**Department of Neurology, Johns Hopkins University, School of Medicine, 5-119 Meyer Building, Baltimore, Maryland 21205 and †Laboratory of Biophysics, IRP, NINCDS, National Institutes of Health, and Marine Biological Laboratory, Woods Hole, Massachusetts 02543*

Abstract

The giant synapse in the squid stellate ganglion has served as a model in the understanding of normal synaptic transmission, but has not been used extensively in the study of changes in external ion concentrations or pharmacological agents. This anomaly is due primarily to the substantial diffusion barrier that exists between the synapse and the bathing medium. The present study describes a technique for the rapid introduction of substances into the synapse by perfusion through the arterial blood supply that improves access by at least 50-fold. This is demonstrated by treatments known to block the Ca^{2+} activated release of transmitter from the nerve terminal: Ca^{2+}-free medium, La^{3+}, and Cd^{2+}. Whereas such treatments take 20 minutes to 3 hours to block transmitter release with bath application, with perfusion they act within a few seconds. The excitatory postsynaptic potential (EPSP) is reduced below action potential threshold in 5 to 22 seconds, and disappears completely in less than a minute. In addition, the use of pressurized O_2 to drive the perfusate through the preparation eliminates the need for superfusion with O_2 and aids in the long term maintenance of the ganglion. This study confirms the important role of Ca^{2+} in the release of transmitter at the giant synapse, and opens up this neurobiologically important preparation for ionic and pharmacologic evaluation.

Introduction

The giant synapse in the stellate ganglion of the squid is a unique preparation for the study of chemical transmission in that both the pre- and postsynaptic axons are of sufficient size to allow microelectrode penetration (Bullock and Hagiwara, 1957). This has allowed a detailed analysis of the physiology of synaptic transmission, in particular the ionic currents associated with the release of transmitter from the presynaptic terminal (*e.g.,* Kusano *et al.,* 1967; Miledi, 1973; Llinas *et al.,* 1981; Charlton *et al.,* 1982). However, relatively little is known about the pharmacological properties of the giant synapse. The principle reason for this appears to be the high diffusion barrier that exists between the external bathing solution and the synapse itself, as noted in previous studies (Bryant, 1958; Webb *et al.,* 1966; Kelly and Gage, 1969; Lester, 1970; Erulkar and Weight, 1977). Attempts to evaluate effects of pharmacological agents or ions on the pre- or postsynaptic axons have been limited or unsuccessful. For example, Bryant (1958) explored the action of a number of pharmacological agents applied to the ganglion via the bathing medium. Only the non-

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Address reprint requests to: E. F. Stanley, NINCDS Laboratory of Biophysics, MBL, Woods Hole, MA 02543.

specific effect of a gradual decline in the excitatory postsynaptic potential (EPSP) was noted and this occurred over a period of 30 minutes or more. Even experiments examining the effect of changes in external concentrations of mono- and divalent cations have been limited by access to the giant synapse. Thus, the diffusion barrier restricted experiments to one or two changes of K^+ concentrations per experiment in a recent study (Erulkar and Weight, 1977) while in other studies the time taken for equilibration of release following changes in external Ca^{2+} was 20 minutes to 3 hours (Miledi and Slater, 1966; Lester, 1970). Other examples in the literature include: 3-aminopyridine (Llinas *et al.,* 1976) and L-glutamate (Kelly and Gage, 1969) and it is likely that the same difficulty has been encountered with other bath applied substances but that these results have never been reported. The long delay between application of a test substance in the bath to its access to the synapse excludes the examination of the acute effects of the agent, and delayed effects are difficult to interpret since there is an increased likelihood that they are due to a secondary factor.

A method of introducing substances through the arterial blood supply of the ganglion is presented in the present report, reducing the access time to the synapse by up to two orders of magnitude. This is illustrated by the rapid actions of Ca^{2+}-free medium, $LaCl_3$, and $CdCl_2$ in blocking synaptic transmission. All of these treatments are known to inhibit the quantal release of transmitter from the nerve terminal by interfering with the voltage triggered influx of calcium ions that normally activates the release mechanism (reviewed by Hagiwara and Byerly, 1981). The experiments described in this study illustrate the improved access of substances into the ganglion with this technique, demonstrate the compatibility of arterial perfusion with recording, and, in addition, present a novel method of oxygenating experimental preparations. Preliminary results of this study have appeared in abstract form (Stanley and Adelman, 1982).

Materials and Methods

Gross dissection

Small squid of less than 14 cm mantle length were selected for study of the giant synapse, since in larger squid the ganglion is less transparent and more difficult to penetrate with microelectrodes.

The initial dissection was similar to that described by Miledi and Slater (1966). The squid was decapitated and the body was immediately placed on a dissection table with fresh oxygenated sea water continuously passing over it. An incision was made up the ventral midline and the mantle was laid flat to reveal the organs of the body (Fig. 1a). The two stellate ganglia lie on the mantle, on either side of the gut muscular wall. The ink sac, gills, reproductive organs, and syphon muscle were removed, and the muscular tube containing the gut was cut up each side. The digestive organ was removed, care being taken not to damage the blood vessels and the pair of nerves from the head ganglia (stellate ganglion pre-nerves) immediately below it (Fig. 1b). The pre-nerves were tied off with cotton thread at their most rostral ends and dissected free from the underlying musculature, back to the holes in the gut muscle where they descend to innervate the ganglia. The muscle overlying the two ganglia was then carefully removed.

Cannulation of the artery

The single medial artery that supplies both ganglia is located between the gut and its muscular wall and can be recognized by its smooth, thick-walled appearance. A

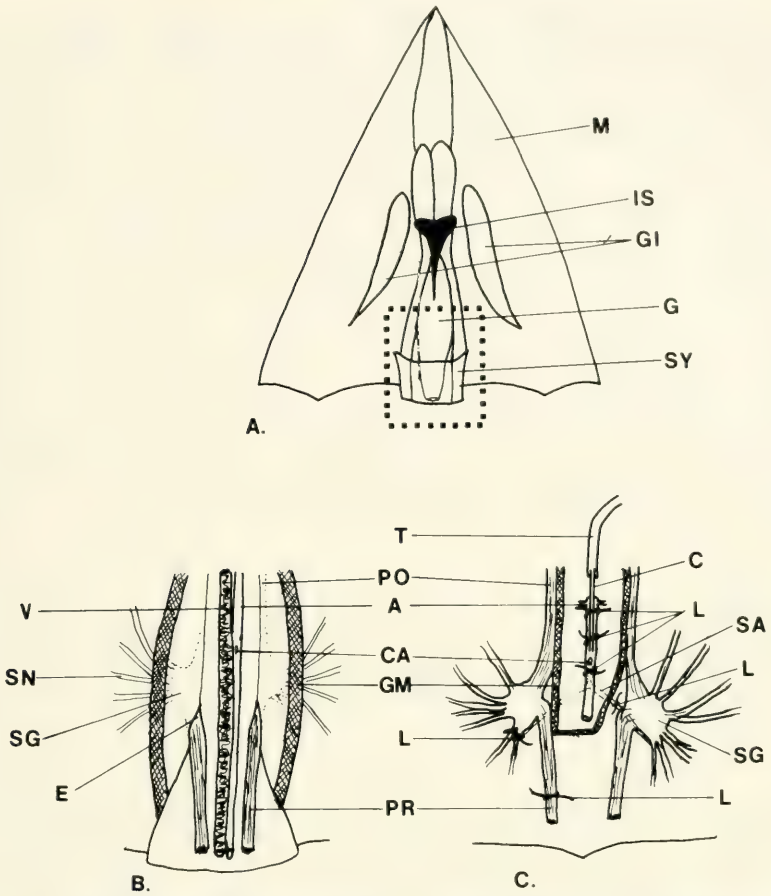

FIGURE 1. Dissection and perfusion technique. A. Gross dissection. The mantle was cut up the midline and reflected to reveal the organs in the mantle cavity. B. Enlarged region denoted by dotted lines in A after removal of the syphon, the ventral gut musculature, and the digestive gland. C. As in B but after removal of the vein, trimming of the remaining gut muscle to reveal the ganglia, and cannulation of the aorta.

M, mantle; IS, ink sack; GI, gill; G, digestive gland; SY, syphon; V, vein; SN, stellar nerve; SG, stellate ganglion; E, point of descent of the prenerve through the digestive gland muscle wall; T, tubing; PO, medial stellar nerve—containing the largest postsynaptic giant axon; CA, point of exit and descent of the common stellate artery from the aorta; GM, cut edge of the digestive gland muscle wall; L, ligature; PR, pre-nerve—containing the presynaptic giant axon; C, cannula; SA, stellate artery.

second, larger and wrinkled vessel is a vein which can be carefully stripped off. The artery supplies both ganglia from a single branch (which we term the common stellate artery) that passes through the underlying muscle and bifurcates to the ganglia (the right and left stellate arteries). After passing the ganglia, the stellate arteries also supply the rostral third of the mantle musculature.

Perfusion of the ganglia requires several ligatures (Fig. 1c). One ganglion, the right, was selected for the present study. The medial aorta was tied off with 6–0 suture just rostral to the common stellate branch. Ligatures were tied loosely at two points on the aorta, caudal to the common stellate artery, in readiness for the cannula. The rostral ligature of this pair was positioned very closely to the common stellate artery

to minimize leakage through small branches that supply the outer gut musculature. The caudal ligature passed through a portion of the underlying muscles. This ligature prevents tension on the cannula from tearing the common stellate artery during the dissection. The medial aorta was then cut approximately 1 cm distal to the common stellar nerve and the cannula—a blunted, 30 g syringe needle connected to PE 10 polyethylene tubing—was inserted and tied in place with its tip directly over the common stellate artery. Two further ligatures were tied: the right stellate artery was tied off proximal to the right ganglion, and the left stellate artery was tied off immediately distal to the left ganglion but proximal to where it bifurcates and descends into the mantle. Other small artery branches may require suturing in individual squid.

The ganglion was dissected free from the mantle by gently lifting the left pre-nerve by means of its ligature, and cutting the membranes that attach it to the mantle. In the process all the nerves emanating from the ganglion were cut except the last (medial) stellar nerve which contains the third order postsynaptic giant axon of interest in this study. This nerve was dissected free for 15 mm and tied off with silk suture. The left ganglion and nerves were then removed, together with a portion of the overlying outer gut muscle between the ganglion and the cannula, and transferred to an experimental chamber, a silastic coated 35-mm petri dish.

Fine dissection

The ganglion was pinned out in the experimental chamber under an atmosphere of O_2 by the threads attached to the pre-nerve and the medial stellar nerve, the remaining piece of gut muscle underlying the cannulated aorta, and by pins through three of the cut stellar nerves.

The stellate ganglion is enveloped by a "capsule" of smooth muscle that continually contracts and relaxes. This capsule must be removed on the upper surface of the ganglion to allow access to the giant synapse. Fortunately, the arteries enter the ganglion from the rear so that cutting a window in the capsule does not affect arterial perfusion of the ganglion. The fin nerve, which passes from the pre-nerve to the medial stellar nerve without entering the ganglion, was left intact except in experiments requiring extracellular recording from the medial stellar nerve. In these experiments a short segment of the fin nerve was removed to allow the recording electrodes to come into close contact with the giant axon. Cutting the fin nerve close to the ganglion was avoided in most cases (but is possible) to avoid damage to, and hence leakage from, the arteries supplying this nerve.

Introducing test substances by changing the perfusion medium

The arterial cannula was connected, via a short length of polyethylene tubing, to a four-way miniature stopcock (Hamilton) that allowed changes in the perfusion medium. Perfusion was carried out by forcing artificial sea water (ASW, composition in m*M*: NaCl, 423.0; KCl, 9.0; $CaCl_2$, 9.3; $MgCl_2$, 22.9; $MgSO_4$, 25.5, Hepes 5.0) through the ganglion with gas pressure from a compressed O_2 tank (12–18 psi). This method of perfusion is of a "constant pressure" type, similar to gravity feed, but allows much greater forces. Constant pressure perfusion was chosen over the alternative of a "constant flow rate" (as with a peristaltic or syringe pump). With the constant pressure method the absolute flow rate is more difficult to control (but was reasonably constant for any preparation) but pressure changes during switching from ASW to test solutions were negligible. This avoided movement artifacts during microelectrode recording. Measured flow rates ranged from 0.1 to 0.5 ml/min, depending in part on leaks through small artery branches that were not ligatured.

An additional major advantage of the perfusion method was the very effective introduction of O_2 into the preparation. Transmission at the giant synapse is known to be highly dependent on an adequate O_2 supply (Bryant, 1958). Since the perfusion solution is forced into the ganglion under a high pressure of O_2, the perfusate is presumably saturated or super-saturated with O_2 when it enters the lower pressure of the ganglion. In fact, with this method it is unnecessary to supply any additional source of O_2 for many hours of study.

The test substance was dissolved in ASW and was then introduced by manually turning the tap on the stopcock. Test solutions were: Ca^{2+} free medium (composition as ASW but $CaCl_2$ 0 m*M*, $MgCl_2$ 65 m*M*); $LaCl_3$ (1 or 3 m*M*); and $CdCl_2$ (1 m*M*). Experiments were carried out at room temperature (22–24°C).

Stimulation and recording techniques

The pre-nerve was stimulated by a square wave pulse of electricity originating from a stimulator (Grass S48) via a stimulus isolation unit (Grass SIU 5) and a pair of silver wire electrodes hooked under the nerve.

Extracellular action potentials in the stellar nerve were recorded by means of a pair of silver wire electrodes connected to a differential amplifier (WPI, DAM6) and a storage oscilloscope (Tektronix D11).

Intracellular recording was carried out by conventional microelectrode techniques. A 3 *M* KCl filled micropipette (5–15 Mohms) was connected via a holder and a probe to a DC amplifier with high input impedance and unit gain (WPI S-7000 system). The output of the amplifier was displayed on the storage oscilloscope (Tektronix D11) to record action potentials and EPSPs, while resting potentials were displayed on the digital meter of the S-7000.

Results

In initial experiments access of perfusate into the ganglion was examined with ASW containing the dye carmine red. Perfusion of dye resulted in a rapid color change of the whole preparation and revealed an interdigitating network of vessels within the ganglion. Dye was detected throughout the region of the ganglion containing nerve fibers and cell bodies and also the first few mm of the pre-nerve and the stellar nerves. A large vessel was usually observed passing very closely to the giant synapse. Fixation of the ganglion by perfusion was also very rapid and resulted in excellent maintenance of morphology (Martin and Miledi, 1975; D. W. Pumplin and E. F. Stanley, unpub. obs.).

In order to examine the access of substances to the giant synapse, it is necessary to select a treatment that is specific for synaptic transmission. This was achieved by blocking transmitter release by two cations known to interfere with calcium flux, La^{3+} or Cd^{2+}, or by Ca^{2+}-free medium.

Effect of La^{3+}. The substantial diffusion barrier to the giant synapse was confirmed by adding $LaCl_3$ (3 m*M*) to the bathing medium of a ganglion incubated without perfusion but with the capsule removed and O_2 continually passing over the preparation. Extracellular stimulation and recording techniques were used to examine transmission across the synapse stimulating the pre-nerve while the postsynaptic giant axon action potential was recorded from the medial stellar nerve. In normal ASW the synapse transmits the impulses for many hours at a stimulus frequency of 1 Hz. After addition of La^{3+}, transmission continued normally for 30 minutes but by 36 minutes the synapse would not transmit 10 consecutive stimuli at 1 Hz, and by 39 minutes there was complete failure.

The effect of perfusing $LaCl_3$ on transmission was compared with that of bath application by use of intracellular recording techniques. The postsynaptic giant axon was penetrated by a microelectrode and a 60 to 70 mV resting potential was recorded. The pre-nerve was stimulated at 1 Hz evoking an EPSP/action potential complex in the postsynaptic giant axon. This preparation was highly stable. The resting potential and synaptic transmission were maintained for several hours of study. La^{3+} (1 m*M*) perfusion resulted in a small decline in the resting potential, failure of the action potential component of the EPSP/action potential complex and a gradual reduction of the EPSP to below the detectable level (Fig. 2). The effect of La^{3+} on the EPSP was irreversible, even after washout periods of 20 minutes, although the RMP did recover. Experiments examining the washout of ions from the preparation were therefore carried out using another ion that blocks the entry of Ca^{2+} into the terminal, Cd^{2+}.

Effect of Cd^{2+}. $CdCl_2$ had a similar effect of $LaCl_3$. The EPSP declined below threshold for an action potential in 7 ± (S.D.) 1 s (n = 4) and to below the level of detection in 14 ± 4 s (Figs. 3a, 4), (minimum 6 s and 12 s, respectively). A decline in the resting potential (10 to 20 mV) was also noted (Fig. 4a), but Cd^{2+} did not block antidromically evoked potentials in the giant axon. Unlike La^{3+}, the effect of Cd^{2+} on synaptic transmission was reversible. Switching the perfusion solution back to ASW resulted in the gradual recovery of the resting potential, and in the reappearance of the EPSP to threshold for an action potential in the postsynaptic axon (Fig. 4b).

Effect of Ca^{2+}-free ASW. The effect of Ca^{2+}-free sea water on synaptic transmission was also examined by intracellular recording from the postsynaptic giant axon. The effect of removing Ca^{2+} was similar to that of the action of La^{3+} or Cd^{2+}, a gradual

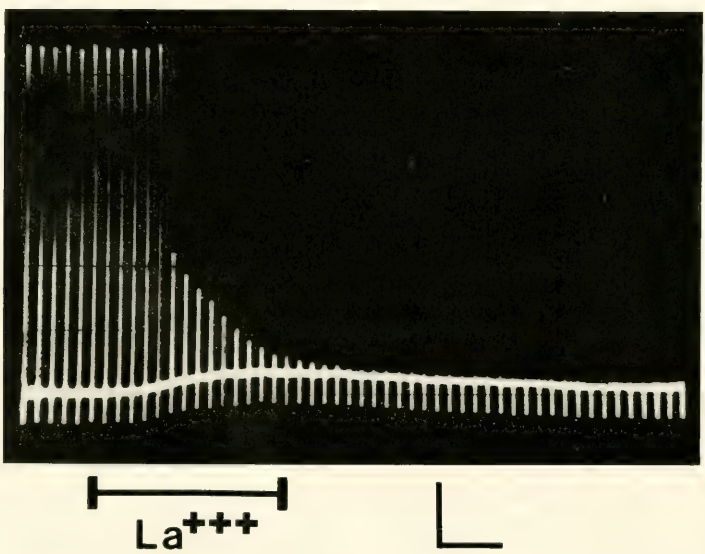

FIGURE 2. Effect of La^{3+} (1 m*M*) infusion (horizontal bar) on potentials recorded intracellularly from the postsynaptic giant axon (stimulus frequency 1 Hz). La^{3+} resulted in a disappearance of the synaptically evoked action potential followed by a decline in the EPSP and a coincident decline in the resting potential. Re-infusing plain ASW (onset at the end of the horizontal bar) resulted in a gradual recovery of the resting potential, but the EPSP did not reappear. The down-going trace deflections are due to a stimulus artifact and obscure the negative after potential of the action potential. Calibration: amplitude = 10 mV, time = 5 s.

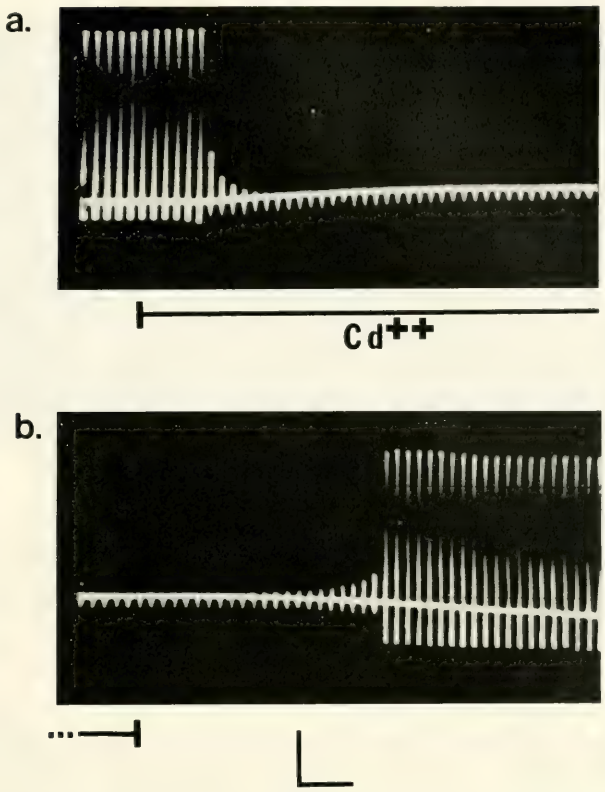

FIGURE 3. Effect of Cd^{2+} on potentials recorded from the postsynaptic giant axon. a. Infusion of Cd^{2+} (1 m*M*) resulted in the disappearance of the synaptically evoked action potential, elimination of the EPSP, and a decline in the resting potential. b. Washout of Cd^{2+} was followed by a reappearance of the EPSP and the action potential and a gradual recovery of the resting potential (retouched). Calibration: amplitude = 20 mV, time = 5 s.

reduction in the EPSP to below threshold and eventually to a complete disappearance (Fig. 5). However, Ca^{2+}-free medium reduced the EPSP at a slower rate than the other treatments, eliminating the synaptically evoked action potential in 13 ± 7 s, and the EPSP in 35 ± 12 s (n = 4) (minimum 5 s and 18 s, respectively). Reperfusion with Ca^{2+}-containing ASW resulted in transmission recovery.

DISCUSSION

The blood system of the squid has been described by Williams (1909), but the arterial system supplying the stellate ganglia was not discussed in detail in this classical study. The heart is situated between the gills in the caudal region of the mantle cavity and the aorta runs rostrally, penetrates the muscular coat of the digestive gland, and passes between the digestive gland muscle wall and the gland itself, terminating in the head. Just rostral to the level of the stellate ganglia the aorta sends a single branch through the digestive gland muscle wall which bifurcates to supply the two stellate ganglia. These arteries continue beyond the ganglia as the major blood supply for the rostral mantle. Thus, cannulation of the aorta together with appropriately positioned

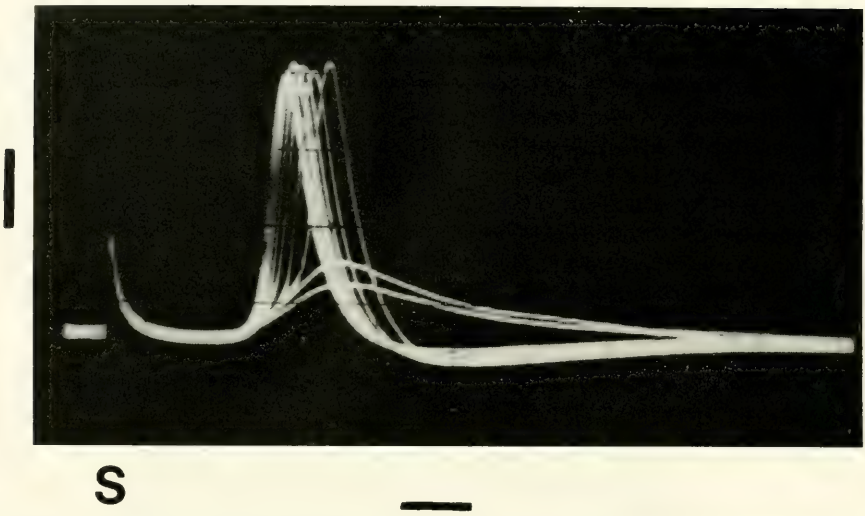

FIGURE 4. Effect of Cd^{2+} infusion on intracellularly recorded potentials from the postsynaptic giant axon. Prior to Cd^{2+} an EPSP/action potential complex was recorded (response with the shortest latency). Infusion of Cd^{2+} resulted in a decline in the EPSP so that threshold, the takeoff point of the action potential, was reached at progressively longer latencies. Transmission failed when the EPSP amplitude declined to below threshold (at 8 s after the onset of Cd^{2+} infusion). S = stimulus artifact; calibration: amplitude = 20 mV, time = 1 ms.

ligatures results in an effective perfusion of one (or both) of the ganglia *in vitro*. This could be demonstrated by simply perfusing dyes or fixative into the preparation.

Treatment with La^{3+} or Cd^{2+}, or removal of Ca^{2+} from the perfusion solution blocked synaptic transmission. The physiological bases for these findings are well established. The action potential in the presynaptic axon terminal triggers the voltage gated influx of Ca^{2+} (Katz and Miledi, 1965) which is believed to result in the fusion of transmitter filled vesicles with the nerve terminal membrane and the discharge of their contents into the synaptic cleft (*c.f.*, Heuser and Reese, 1973). The cations La^{3+} (Heuser and Miledi, 1971) and Cd^{2+} (Satoh *et al.*, 1982) are known to block Ca^{2+} entry into the nerve terminal (see Hagiwara and Byerly, 1981, for recent review). The effects of these two ions on the EPSP were similar, except that La^{3+} was irreversible, as in other preparations (Heuser and Miledi, 1971). Perfusion of La^{3+} or Cd^{2+} into the ganglion resulted in a decline in the EPSP with complete blockade of transmission in a minimum of 12 seconds. In fact, this latency does not take into consideration the time taken to clear the dead space in the cannula after switching from ASW to ASW plus the test ion, typically 3 to 4 seconds. Thus, arterial perfusion can block Ca^{2+} triggered transmitter release within 9 seconds of introduction, an access improvement of at least 100-fold.

Ca^{2+}-free medium had a similar effect to that of La^{3+} and Cd^{2+}. As reported previously removal of Ca^{2+} from the external medium eliminates the release of transmitter at the giant synapse (Miledi and Slater, 1966; Lester 1970). However, transmission is blocked at least 50 times faster with perfusion than by removing Ca^{2+} from the bathing medium, as reported in these previous studies. Re-introducing Ca^{2+} in ASW results in recovery of transmission, indicating that with the brief exposures necessary with the perfusion technique Ca^{2+}-free medium does not permanently damage the nerve terminal.

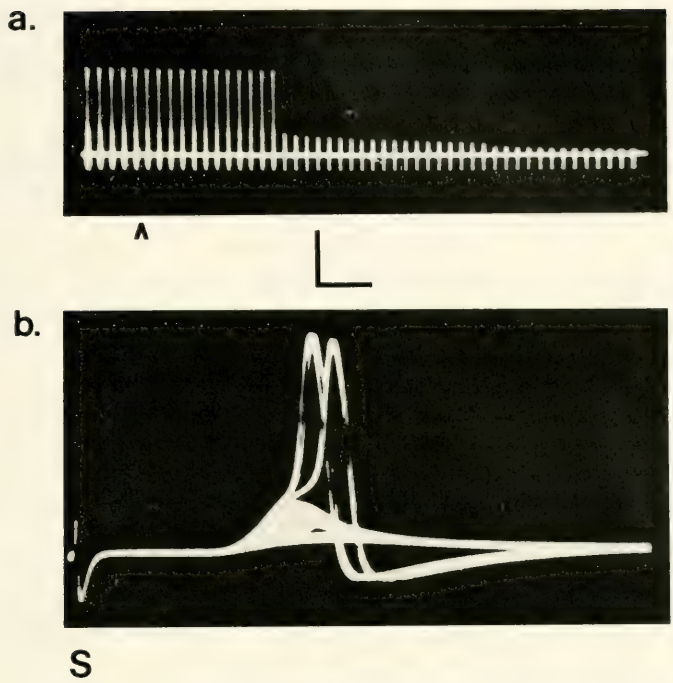

FIGURE 5. Effect of Ca^{2+}-free infusion on synaptic transmission at the giant synapse. a. Infusion of Ca^{2+}-free ASW (onset at arrow head) resulted in a failure of the action potential component of the intracellularly recorded evoked response in the postsynaptic giant axon followed by the gradual disappearance of the EPSP. Calibration: 50 mV, 5 s. b. Same preparation as in (a) at a fast trace sweep speed. The superimposition of EPSPs that are gradually declining amplitude obscures the visualization of individual traces. Calibration 20-mV, time = 1 ms.

Both La^{3+} and Cd^{2+} also had a limited depolarizing effect on the resting potential of the postsynaptic axon. The basis for this effect is not well established. Cd^{2+} has been reported to reversibly slow the activation kinetics of voltage sensitive K^+ channels (Gilley and Armstrong, 1982). If some of the K^+ channels were open at rest, their closure would result in a decline in the resting potential. Cd^{2+} and La^{3+} may also reduce the resting potential by direct effects on membrane ion permeability, for example La^{3+} increases the flux of Na^+ ions (P. DeWeer, pers. comm.).

The results of this study show that ion penetration into the stellate ganglion is greatly enhanced by arterial perfusion. Clearly substances can gain quick access to a *small fraction* of the giant synapse from the bathing medium; for example Ca^{2+} iontophoresed on to the presynaptic terminal in Ca^{2+}-free medium is capable of triggering limited transmitter release (Miledi and Slater, 1966). However, a substantial fraction of the presynaptic terminal must be inaccessible to bath application, as discussed above. The usefulness of the perfusion technique is that the ions must gain access to the entire synapse or it would not be possible to completely eliminate transmitter release. Similar arguments can be made for the postsynaptic giant axon where bath-applied glutamate fails to depolarize even after prolonged exposure at high concentrations (Kelly and Gage, 1969), whereas perfusion results in a depolarization that is detectable within seconds (Stanley, 1983, 1984).

Many other substances can also be introduced into the giant synapse by this

technique. Recently perfusion has been used to demonstrate and characterize a cholinergic receptor on the postsynaptic giant axon, and to compare the actions of a variety of glutaminergic and cholinergic agonists (Stanley, 1983, 1984). In addition, the perfusion technique is compatible with intracellular recording from the presynaptic giant axon (Stanley, 1984). The latter is perhaps the most exciting application for this technique: the examination of the effects of ions and pharmacological agents on the unique aspect of squid giant synapse, the accessible presynaptic giant axon terminal.

ACKNOWLEDGMENTS

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LITERATURE CITED

BRYANT, S. H. 1958. Transmission in squid giant synapses: the importance of oxygen and the effect of drugs. *J. Gen. Physiol.* **41:** 473–484.

BULLOCK, T. M., AND S. HAGIWARA. 1957. Intracellular recording from the giant synapse of the squid. *J. Gen. Physiol.* **40:** 565–577.

BULLOCK, T. H. 1948. Properties of a single synapse in the stellate ganglia of the squid. *J. Neurophysiol.* **42:** 609–616.

CHARTON, M. P., S. J. SMITH, AND R. S. ZUCKER. 1982. Role of presynaptic calcium ions and channels in synaptic facilitation and depression at the squid giant synapse. *J. Physiol.* **323:** 173–193.

ERULKAR, S. D., AND F. F. WEIGHT. 1977. Extracellular potassium and transmitter release at the giant synapse of squid. *J. Physiol. (Lond.)* **266:** 209–218.

GILLEY, W. F., AND C. M. ARMSTRONG. 1982. Divalent cations and the activation kinetics of potassium channels in squid axons. *J. Gen. Physiol.* **79**(2): 965–996.

HAGIWARA, S., AND L. BYERLY. 1981. Calcium channel. *Ann. Rev. Neurosci.* **4:** 69–125.

HEUSER, J. G., AND R. MILEDI. 1971. Effect of lanthanum ions on structure and function of frog neuromuscular junctions. *Proc. R. Soc. Lond. B* **179:** 247–260.

HEUSER, J. G., AND T. S. REESE. 1973. Evidence for recycling of synaptic vesicle membrane during transmitter release at the frog neuromuscular junction. *J. Cell. Biol.* **57:** 315–344.

KATZ, B., AND R. MILEDI. 1965. The effect of calcium on acetylcholine release from motor nerve terminal. *Proc. R. Soc. Lond. B* **161:** 196–503.

KELLY, J. S., AND P. W. GAGE. 1969. L-glutamate blockade of transmission at the giant synapse of the squid stellate ganglion. *J. Neurobiol.* **2:** 209–219.

KUSANO, K., D. R. LIVENGOOD, AND R. WERMAN. 1967. Correlation of transmitter release with membrane properties of the presynaptic fiber of the squid giant synapse. *J. Gen. Physiol.* **50:** 2579–2601.

LESTER, M. A. 1970. Transmitter release by presynaptic impulses in the squid stellate ganglion. *Nature* **227:** 493–496.

LLINAS, R. L., I. Z. STEINBERG, AND K. WALTON. 1981. Relationship between presynaptic calcium current and postsynaptic potential in squid giant synapse. *Biophys. J.* **33:** 323–352.

LLINAS, R., K. WALTON, AND V. BOHR. 1976. Synaptic transmission in squid giant synapse after potassium conductance blockade with external 3- and 4-aminopyridine. *Biophys. J.* **16:** 83–86.

MARTIN, R., AND R. MILEDI. 1975. A presynaptic complex in the giant synapse of the squid. *J. Neurocytol.* **4:** 121–129.

MILEDI, R. 1973. Transmitter release induced by injection of calcium ions into the nerve terminal. *Proc. R. Soc. Lond. B* **183:** 421–425.

MILEDI, R., AND SLATER. 1966. The action of calcium on the neuronal synapses in the squid. *J. Physiol.* 184, 473–498.

SATOH, E., A. FUMITOSHI, I. KATSUAKI, AND M. NISHIMURA. 1982. Mechanisms of cadmium induced blockade of neuromuscular transmission. *Eur. J. Pharmacol.* **77:** 251–257.

STANLEY, E. F. 1983. Depolarizing and desensitizing actions of glutaminagic and cholinergic agonists at the squid giant synapse. *Biol. Bull.* **165:** 533.

STANLEY, E. F. 1984. Action of cholinergic agents on the squid giant synapse. *J. Neurosci.* **4:** 1904–1911.

STANLEY, E. F., AND W. J. ADELMAN, JR. 1982. Perfusion of the squid stellate ganglion through its blood supply: Implications for morphological and physiological studies of the squid giant synapse. *Biol. Bull.* **163:** 403.

WEBB, G. D., W. D. DETTBARN, AND M. BRISIN. 1966. Biochemical and pharmacological aspects of the synapses of the squid stellate ganglia. *Biochem. Pharmacol.* **15:** 1813–1814.

WILLIAMS, L. W. 1909. *The Anatomy of the Common Squid.* Brill, Leiden, Holland. 92 pp.

Reference: *Biol. Bull.* **167**: 477–487. (October, 1984)

DAILY BUDGETS OF PHOTOSYNTHETICALLY FIXED CARBON IN SYMBIOTIC ZOANTHIDS

R. GRANT STEEN AND L. MUSCATINE

Department of Biology, University of California, Los Angeles, California 90024

ABSTRACT

We tested the hypothesis that some zoanthids are able to meet a portion of their daily respiratory carbon requirement with photosynthetic carbon from symbiotic algal cells (= zooxanthellae). A daily budget was constructed for carbon (C) photosynthetically fixed by zooxanthellae of the Bermuda zoanthids *Zoanthus sociatus* and *Palythoa variabilis*.

Zooxanthellae have an average net photosynthetic C fixation of 7.48 and 15.56 $\mu gC \cdot polyp^{-1} \cdot day^{-1}$ for *Z. sociatus* and *P. variabilis* respectively. The C-specific growth rate (μ_c) was $0.215 \cdot day^{-1}$ for *Z. sociatus* and $0.152 \cdot day^{-1}$ for *P. variabilis*. The specific growth rate (μ) of zooxanthellae in the zoanthids was measured to be 0.011 and $0.017 \cdot day^{-1}$ for *Z. sociatus* and *P. variabilis* zooxanthellae respectively. *Z. sociatus* zooxanthellae translocated 95.1% of the C assimilated in photosynthesis, while *P. variabilis* zooxanthellae translocated 88.8% of their fixed C. As the animal tissue of a polyp of *Z. sociatus* required 14.75 $\mu gC \cdot day^{-1}$ for respiration, and one of *P. variabilis* required 105.54 $\mu gC \cdot day^{-1}$, the contribution of zooxanthellae to animal respiration (CZAR) was 48.2% for *Z. sociatus* and 13.1% for *P. variabilis*.

INTRODUCTION

Zoanthids (Cnidaria:Zoanthidea) are conspicuous and abundant members of shallow intertidal reef flat communities where scleractinian coral growth is not extensive (Sebens, 1982). Zoanthids contain dense populations of symbiotic algae (=zooxanthellae) which can fix carbon and translocate photosynthetic products to the animal host (Von Holt and Von Holt, 1968; Trench, 1974). Sebens (1977) has recently described the natural diet of the zoanthids *Zoanthus sociatus* and *Palythoa variabilis* at study sites in Panama and Brazil. Although the species exist in close proximity to each other, *P. variabilis* fed extensively on demersal zooplankton while *Z. sociatus* fed sparingly on small detrital particles. Sebens (1977) also demonstrated that although *Z. sociatus* lost weight when starved, significantly less weight was lost when starved in light sufficient for photosynthesis by symbiotic zooxanthellae. Because *Z. sociatus* fed on fewer, smaller food items than *P. variabilis*, Sebens (1977) conjectured that *Z. sociatus* polyps acquired a larger proportion of their daily carbon requirement from zooxanthellae than did *P. variabilis* polyps.

Using a new technique for analysis of daily C budgets in symbiotic cnidarians (Muscatine *et al.*, 1983; Muscatine *et al.*, 1984) it is now possible to obtain accurate estimates of at least four parameters associated with daily flux of photosynthetically fixed carbon: (1) the total daily net C fixed by zooxanthellae, (2) the amount of C used in growth of zooxanthellae, (3) the amount translocated to the animal, (4) and the contribution of translocated C to animal respiration.

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This technique has been applied to symbiotic corals and has revealed that up to 97% of the carbon fixed in photosynthesis is translocated to the animal where it can potentially satisfy not only 100% of the animal daily requirement for respiration, but also a fraction of the carbon required for growth (Muscatine *et al.*, 1984).

In this paper we analyze the daily budget of photosynthetically fixed carbon in the zoanthids *P. variabilis* and *Z. sociatus* from shallow waters of Bermuda. We quantitatively assess the importance of photosynthetically fixed carbon to these animals, and compare our findings to previous work on zoanthid nutrition.

Materials and Methods

Collection and maintenance of animals

Zoanthus sociatus and *Palythoa variabilis* were collected from Hungry Bay, Paget Parish, Bermuda at depths ranging from 0–0.3 m. The larger *P. variabilis* (2–4 cm high; 0.5–1.5 cm wide) were scattered among the polyps of the much smaller (0.5–1.5 cm high; 0.2–0.6 cm wide) and more abundant *Z. sociatus*. Small rocks with attached zoanthids were dislodged and transported in covered collecting buckets to the laboratory. Zoanthids were maintained in a 200 l plexiglass sea water tank (water flow rate = 2.4 $l \cdot min^{-1}$) under natural sunlight outdoors. All animals were kept for a day in the tank and used in experiments within four days. To determine if maintenance conditions were adequate, the behavior of zoanthids in the tank was regularly monitored. The number of polyps fully or partially open per 1000 animals was counted for each species at 1–2 hour intervals over 24 hours.

Standing stock of zooxanthellae cell carbon (C')

Sixty-five randomly selected polyps of *Z. sociatus* were individually homogenized in a Waring blender equipped with a micro-attachment. Twelve randomly selected *P. variabilis* polyps were similarly treated. The homogenates were centrifuged at low speed and the resulting algal pellet resuspended in sea water. Numbers of zooxanthellae in the pellet were counted from samples using a Spencer Bright-Line haemacytometer. The diameter of 500 freshly isolated zooxanthellae was determined with an ocular micrometer and used to calculate average cell carbon according to Strathmann (1967). Standing stock of algae is expressed as number of cells per polyp, or as algal carbon per polyp.

Photosynthesis versus *irradiance curves of zooxanthellae*

Total C fixed daily was determined from measurements of photosynthetic rates at various irradiances using the ^{14}C technique (Strickland and Parsons, 1977). Individual zoanthid polyps were clipped from colonies and allowed two days to heal before use. Healthy polyps were placed in conical centrifuge tubes in 1 ml of Millipore filtered (0.45 μm) seawater. Tubes were wrapped with plastic neutral-density screens and placed in front of a bank of Cool-White fluorescent lights to obtain a range of irradiances from 0–360 μ Einsteins $\cdot m^{-2} \cdot s^{-1}$. Six ml of a stock solution of $NaH^{14}CO_3$ in filtered sea water was added to each tube so that the total dose was 1 μCi per tube. Three 0.1 ml samples were taken immediately for measurement of added activity. Polyps were incubated 1 hour at 27–28°C, followed by addition of 0.5 ml of 40% formalin to each tube and refrigeration at 5°C before further processing. Subsequently each polyp was individually homogenized and the zooxanthellae quantitatively isolated as

before. The animal homogenate was adjusted to 12 ml and a 0.4 ml aliquot was placed in a scintillation vial, acidified with 1 *N* HCl, and evaporated to dryness at low heat on a Waring hot plate to remove unused $^{14}CO_2$. Ten ml of Aquasol was added to the dried sample and radioactivity was measured in a Beckman LS-100C liquid scintillation counter using a ^{14}C wide window isoset.

Algal pellets were resuspended in 1 ml of Millipore filtered (0.45 μ) sea water. A 0.5 ml aliquot was filtered, rinsed, acidified, and counted in 10 ml Aquasol. The remainder of the algal suspension was saved for haemacytometer counts.

Scintillation counts were quench corrected and disintegrations per minute (dpm) in the animal and algal fractions were summed to obtain total dpm per polyp. Total weight of carbon fixed was calculated (Strickland and Parsons, 1977), corrected for dark fixation, and expressed as net carbon fixed. Data were normalized to the number of zooxanthellae counted in individual animals and regressed against irradiance. Photosynthetic capacity (P_{max}) was calculated as the average of the total carbon fixed in all experiments at or above saturation.

Total daily net photosynthesis of zooxanthellae was calculated from the hourly rate of photosynthesis at saturation multiplied by the number of hours each zoanthid was at saturating irradiance.

Natural ambient irradiance was measured on 8 September 1983 with a LiCor 185 light meter equipped with a quantum sensor. The sensor was taped to an unshaded horizontal surface and readings were taken at intervals from 6:15 AM to 8:00 PM.

Specific growth rate of zooxanthellae

The carbon specific growth rate (μ_c) was calculated from:

$$\mu_c = \frac{1}{C'} \cdot \frac{\Delta C}{\Delta t}$$

where C′ is the standing stock of algal carbon, and $\Delta C/\Delta t$ is the increment of C added per day (= net carbon fixed per day).

Cell specific growth rate (μ) was estimated by the method of Wilkerson *et al.* (1983), using zooxanthellae harvested from each zoanthid species at 2 hour intervals for 24 hours. The algal pellet was preserved in 4% formalin in sea water and the number of cells in cytokinesis (*i.e.*, a "doublet" with a cell wall plate) was counted in each of 10 fields of 200 zooxanthellae viewed at 430×. The specific growth rate (μ) of zooxanthellae was calculated from:

$$\mu = \frac{1}{t_d} \ln(1 + f)$$

where f is the average mitotic index, and t_d is the duration of mitosis, taken here as 0.46 days (McDuff and Chisholm, 1983; Wilkerson *et al.*, 1983).

Translocation of carbon from zooxanthellae to host

Fixed carbon translocated daily from zooxanthellae was estimated by the growth rate method (Muscatine *et al.*, 1983) such that $\mu_c - \mu = \mu_T$ where μ_T is the specific translocation rate and T is the percent total daily translocation, such that:

$$T = \frac{\mu_c - \mu}{\mu_c} \times 100$$

Respiration

Respiration of the intact plant-animal association was measured by placing freshly collected polyps of each species separately in 5-l Niskin sampling bottles (General Oceanics Model 1010) filled with sea water from the running sea water system. Bottles were incubated for 12 hours in darkness at 27–28°C, then water from the Niskin bottles was run into replicate BOD bottles. Dissolved oxygen was measured in the water before and after incubation using the Winkler technique (Strickland and Parsons, 1977).

To estimate the respiration of the animal component we assumed that the ratio of plant to animal respiration was proportional to their protein biomass ratio (Muscatine *et al.*, 1981). The protein content of freshly separated plant and animal tissues in each polyp was measured after incubation using the method of Lowry *et al.* (1951) with bovine serum albumin as a standard.

The daily contribution of zooxanthellae to animal respiration (CZAR)

Since daily translocation represents the carbon supply and daily animal respiration the carbon demand, CZAR was calculated from:

$$\text{CZAR} = \frac{\text{Net C fixed daily}}{R_a} \cdot T$$

where R_a = total daily animal respiratory requirement for carbon (Muscatine *et al.*, 1983).

Results

Expansion and contraction of polyps

To determine if maintenance and incubation conditions were adequate to sustain normal behavior patterns, we compared the pattern of expansion and contraction of polyps of both zoanthid species. Figure 1 shows that as many as 75% of the polyps of *P. variabilis* were fully or partially expanded in darkness, while only 1–4% of the polyps were expanded in full sunlight. In contrast, those of *Z. sociatus* showed an irregular pattern of expansion and contraction, with groups of polyps showing similar behavior. *Z. sociatus* polyps did not show a marked response to light, but contracted rapidly in response to wind-driven water disturbances.

These patterns of expansion and contraction of zoanthids in the holding tank were similar to those seen by Sebens (1977) for these species in the field, and persisted until at least four days after collection, suggesting that maintenance conditions did not perturb normal expansion and contraction behavior.

Standing stock of zooxanthellae

Table I shows that *Z. sociatus* polyps harbor an average of 1.11×10^6 zooxanthellae, while polyps of the larger *P. variabilis* contain 52% more zooxanthellae. Since zooxanthellae were isolated collectively from a group of zoanthids, the range of variation in zooxanthellae per polyp is unknown.

Zooxanthellae isolated from *P. variabilis* were significantly larger (2-sample *t*-test, $P < 0.0005$) and had more than twice the volume and twice the cell carbon than zooxanthellae from *Z. sociatus.* Polyps of the larger *P. variabilis* contained almost three times as much algal carbon as those of *Z. sociatus.*

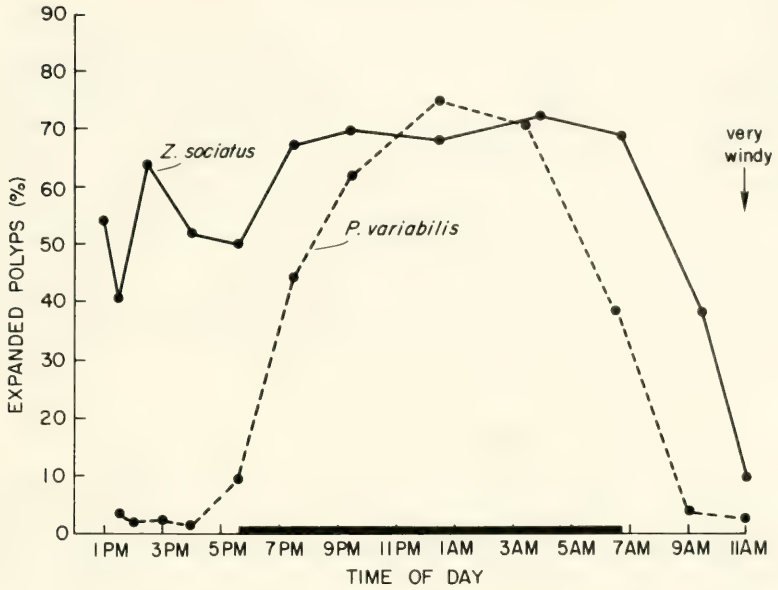

FIGURE 1. The diel pattern of polyp expansion of zoanthids in the outdoor aquarium. Note similarity to behavior in the field reported by Sebens (1977). Arrow indicates first observation time affected by a gusting wind which began shortly after 9 AM.

Photosynthesis/irradiance curves for intact zoanthids

Figure 2 shows the photosynthesis/irradiance (P/I) curves derived for both zoanthid species. The P/I curve for *Z. sociatus* is linear up to at least 360 μ Einsteins $\cdot m^{-2} \cdot s^{-1}$ and may extend beyond this point before saturating. Consequently, our estimate for P_{max} may well be conservative. During the $^{14}CO_2$ incubation these polyps randomly expanded and contracted as did their counterparts in the holding tank. Zooxanthellae were therefore exposed to irradiance that was attenuated by an animal body wall of variable thickness. This may account for the lower correlation coefficient.

The P/I curve for *P. variabilis* is linear up to about 180 μ Einsteins $\cdot m^{-2} \cdot s^{-1}$. There was relatively little variation in photosynthesis at a given irradiance. During the $^{14}CO_2$ incubation these zoanthids were contracted and showed little change in posture. Thus the zooxanthellae in *P. variabilis* may have received a more constant light flux penetrating the body wall than in *Z. sociatus* and the rate of photosynthesis would not be affected by changes in body wall thickness.

TABLE I

Biomass parameters of zoanthids

Zoanthid	Zooxanthellae cells $\cdot$ polyp^{-1} ($\times 10^6$)	Zooxanthellae diameter (μm $\pm$ S.D.)	Zooxanthellae cell volume (μm^3)	Zooxanthellae carbon $\cdot$ cell^{-1} (pg)	Zooxanthellae C $\cdot$ polyp^{-1} (μg)
Z. sociatus	1.11	7.025 $\pm$ 0.79	181.5	31.35	34.80
P. variabilis	1.69	9.050 $\pm$ 3.42	388.2	60.56	102.35

Daily net carbon fixed

Figure 3 shows the measured irradiance at the Bermuda Biological Station on 8 September 1983, a clear cloudless day. This curve of light available for photosynthesis on a "representative day" was used in conjunction with the P/I curves to calculate the total daily increment of carbon fixed by zooxanthellae photosynthesis. Our estimate of absolute values of net daily photosynthesis is strictly valid only for the day on which the diel irradiance curve was recorded, but it reflects the potential for any day. The method also assumes that photosynthesis at dawn and dusk, at levels below P_{max}, does not significantly contribute to total diel C fixation.

Table II shows net hourly C fixation at P_{max} normalized to 10^6 zooxanthellae and to an average polyp of each species. The data show that *P. variabilis* fixed twice as much carbon per polyp per day as *Z. sociatus*.

Cell specific growth rate of zooxanthellae

Figure 4 shows the average number of dividing zooxanthellae per 200 zooxanthellae examined for each host. Zooxanthellae in both hosts were characterized by a low specific growth rate with an average of 0.63% algal cells in division. Zooxanthellae of *Z. sociatus* showed no phasing of cell division, but *P. variabilis* zooxanthellae showed slight phasing of cell division beginning at 11 PM. The number of dividing cells among *P. variabilis* zooxanthellae is significantly higher (two sample *t*-test; $P < 0.01$) than for *Z. sociatus* zooxanthellae in several of the night time samples. The growth rate of *Z. sociatus* zooxanthellae was 0.0105 day^{-1} (doubling time = 66 days) and for *P. variabilis* $\mu = 0.0170$ day^{-1} (doubling time = 41 days) (Table III).

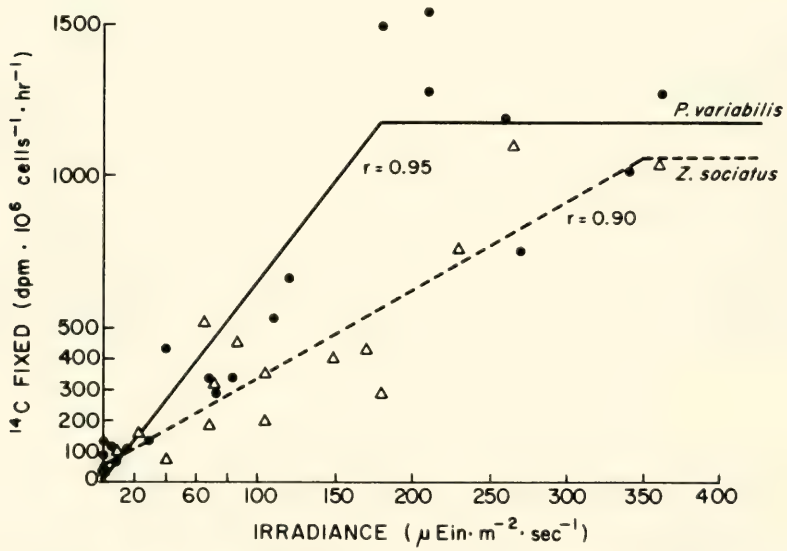

FIGURE 2. The photosynthetic carbon-14 fixation of intact zoanthids *versus* irradiance. The lower linear portion of each curve was fitted by linear regression with data points added in order of increasing irradiance. Correlation coefficients (r) were calculated for each new data point and the point at which the r value began to decline progressively was taken as the last point on the linear portion of the curve. Photosynthesis at saturation (P_{max}) was calculated as the average of the remaining points.

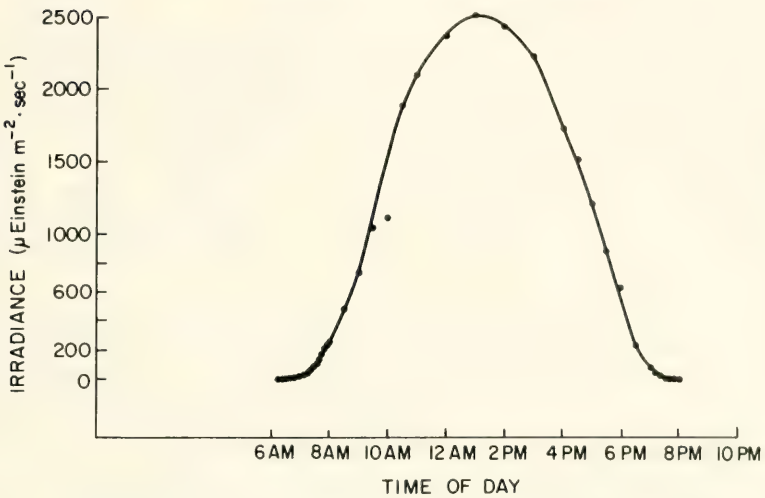

FIGURE 3. Diel irradiance at the Bermuda Biological Station on 8 September 1983, a clear cloudless day.

Translocation

The fraction of C translocated from zooxanthellae is shown in Table III, and the amount of C translocated is calculated from the product of net C fixed daily (Table II) and the percent translocation (Table III). Carbon translocated is substantial in both cases, but greater in *Z. sociatus.*

Respiration

From data on polyp respiration and protein biomass ratios (Table IV) we calculated the average daily animal respiration rate per polyp (Table V). Although the rate of respiration by the larger *P. variabilis* polyps is more than six times that of *Z. sociatus,* their specific metabolic rates are similar (Table V).

Contribution of zooxanthellae to animal respiration (CZAR)

From data on weight of C translocated, and weight of C required for animal respiration we calculated CZAR. CZAR is nearly 50% in *Z. sociatus* polyps but only 13% in *P. variabilis* polyps (Table V).

TABLE II

Total carbon fixed daily by zooxanthellae

Zoanthid species	Saturating irradiance (μEin · $m^{-2} \cdot s^{-1}$)	Net hourly C fixation at P_{max} ($\mu g \cdot 10^6$ $cells^{-1} \cdot h^{-1}$)	Net hourly C fixation at P_{max} ($\mu g \cdot polyp^{-1} \cdot h^{-1}$)	Time at P_{max} (hours)	Net daily C fixation ($\mu g \cdot 10^6$ $cells^{-1} \cdot day^{-1}$)	Net daily C fixation ($\mu g \cdot polyp^{-1} \cdot day^{-1}$)
Z. sociatus	360	0.65	0.72	10.3	6.74	7.48
P. variabilis	180	0.86	1.45	10.7	9.21	15.56

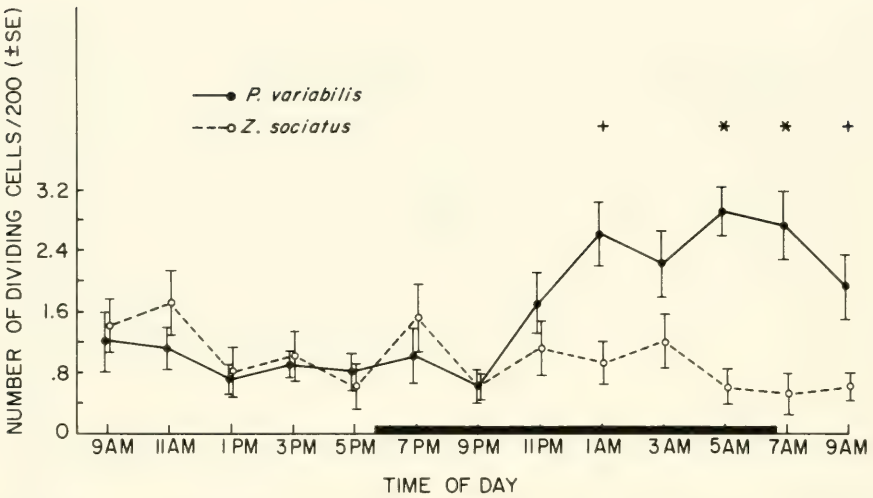

FIGURE 4. Diel variation in number of dividing cells per 200 zooxanthellae. Dividing cells are defined as paired cells or doublets with a division plate between nuclei. Points shown are the mean of 10 counts of cells ± SEM. Significant differences between zooxanthellae from the two zoanthid hosts are seen at several times; levels of significance are (+) $P < .05$ and (*) $P < .001$ (two-sample t-test).

DISCUSSION

The results of this investigation show that zooxanthellae of *P. variabilis* and *Z. sociatus* assimilate substantial amounts of carbon during photosynthesis. The surplus beyond that needed for zooxanthellae respiration and growth can be translocated to the zoanthid host and used to satisfy a portion of the daily animal respiratory requirement for carbon. Our data suggest that zooxanthellae of *Z. sociatus* may contribute 48.2% of the daily animal respiratory requirement for carbon, while zooxanthellae of *P. variabilis* can contribute only 13.1% We conclude that, in order to meet its daily C requirement for respiration, *P. variabilis* is dependent to a greater extent upon feeding than *Z. sociatus*. This conclusion supports that of Sebens (1977).

Both *Z. sociatus* and *P. variabilis* feed on minute particles suspended in the water column. *P. variabilis* obtains demersal zooplankton by tentacular feeding at night while *Z. sociatus* polyps are continuously expanded and feed on small detrital particles rather than whole zooplankton (Sebens, 1977). The greater dependence of *P. variabilis* on capture of demersal zooplankton is correlated with nocturnal polyp expansion behavior (Fig. 1), elongated tentacles, and a tall, columnar body (Koehl, 1977). *Z. sociatus*, which is less dependent upon zooplankton, expands irregularly (Fig. 1), and

TABLE III

Growth rates and translocation by zoanthid zooxanthellae

Zoanthid species	μ_c (day^{-1})	μ (day^{-1})	C translocated daily (%)	C translocated daily ($\mu g \cdot polyp^{-1} \cdot day^{-1}$)
Z. sociatus	0.215	0.011	95.11	7.11
P. variabilis	0.152	0.017	88.80	13.82

TABLE IV

Protein biomass ratios of zoanthids

Zoanthid species	Zooxanthellae protein · polyp^{-1} (mg)	Total protein · polyp^{-1} (mg)	Protein biomass ratio (animal:total protein)
Z. sociatus	0.388	2.268	0.829
P. variabilis	0.516	14.706	0.965

possesses short tentacles and a matting colony morphology which is less suitable for raptorial zooplankton capture (Porter, 1976; Koehl, 1977; Sebens, 1977). The amount of carbon derived from algal photosynthate thus bears an inverse relation to the dependence on heterotrophic C acquisition.

We note that the rates of translocation determined here by the growth rate method are the highest yet reported for symbiotic zoanthids. They are substantially higher than rates reported by Trench (1974) whose analysis was based on the *in vitro* ^{14}C method. These and other methods are critically reviewed in Muscatine *et al.* (1984).

Error in the estimate of P_{max} will substantially change the calculated value for the increment of carbon fixed per day. The P_{max} value for *P. variabilis* is the average of six data points and shows light saturation at irradiances higher than 180 μ Einstein $m^{-2} \cdot s^{-1}$. The P/I curve for *Z. sociatus* does not show a clearcut point at which light saturation begins and it is possible that zooxanthellae in this host were not saturated even at 360 μ Einstein $\cdot m^{-2} \cdot s^{-1}$. If this is true, then *Z. sociatus* zooxanthellae may be capable of fixation of a larger increment of carbon per day than our figures show, and CZAR for this host may be higher than 48.2%. CZAR varies directly with P_{max} such that, all other values being constant, CZAR will increase by 1% for every 1% increase in P_{max}.

The quantitative assessment of P_{max} is conservative because it was measured using a radiotracer technique. Although ^{14}C measurements of plant productivity often overestimate net C uptake (Peterson, 1980), Smith (1982) has pointed out that, in short-term experiments (*e.g.*, 30′–120′), ^{12}C bicarbonate/^{14}C-bicarbonate disequilibrium may result in underestimates of C release by more than 50%.

A major assumption of the carbon budget analysis is that the net carbon assimilated by algal photosynthesis is used only for algal growth or translocation to the host. Other carbon sinks may exist which are not incorporated into this analysis. If zooxanthellae are capable of intracellular storage of significant quantities of fixed carbon our estimate of daily carbon translocation will be high. Storage, however, should

TABLE V

Respiration and CZAR in zoanthids

Zoanthid species	Polyp respiratory rate ($\mu g\ O_2 \cdot day^{-1}$)	Animal respiratory rate ($\mu g\ O_2 \cdot day^{-1}$)	Animal specific metabolic rate ($\mu g\ O_2 \cdot mg\ protein^{-1} \cdot day^{-1}$)	Animal C requirement[1] (μg)	$\frac{\text{C supplied}}{\text{C required}}$ (%)
Z. sociatus	56.92	47.19	25.1	14.75	48.2
P. variabilis	350.00	337.75	23.8	105.54	13.0

[1] Respiratory quotient = 0.8.

produce a change in zooxanthellae volume which was not observed in our samples (Goto *et al.*, pers. commun.). If zoanthids are able to digest zooxanthellae, our estimate of CZAR may be too low.

Determination of the animal daily carbon requirement for respiration depends upon the assumption that the protein biomass ratio of algal and animal components is proportional to their respiration ratio (Muscatine *et al.*, 1981) (*i.e.*, that the specific metabolic rate of the algal and animal tissue is equal). This assumption is difficult to test in intact associations. It is noteworthy that the dark respiration rate of freshly isolated zooxanthellae of the coral *Stylophora pistillata* is nearly 8 times higher than the calculated respiration rate for algae *in situ* (McCloskey and Muscatine, 1984). Some insight may be gained from measurement of the respiratory rate of aposymbiotic hosts, but such hosts would not have translocated substrates to metabolize and therefore would not reflect conditions in the intact association.

A four-fold difference in CZAR exists between *Z. sociatus* and *P. variabilis*. This difference is correlated with a five-fold difference in biomass ratio. A correlation between CZAR and biomass ratio may be a general feature of coelenterate symbioses since it has been observed in several coral and anemone associations (G. Parker, pers. comm.). If a partially heterotrophic organism is to increase its reliance on autotrophic C, either the proportion of space allotted to algae must increase or the metabolic rate of the heterotroph must decrease. The specific metabolic rate of animal tissue in the two zoanthid species is equivalent (Table V), suggesting that *Z. sociatus* can rely more on autotrophic C acquisition because it has proportionally more autotrophic tissue. The protein biomass ratio of *Z. sociatus* is unusually low compared to the biomass ratios of 0.86–0.97 reported for other symbiotic cnidarians (Muscatine, 1980).

Although the work reported here provides evidence for potential quantitative benefit to zoanthids which maintain algal endosymbionts, it does not demonstrate that this potential is realized. When considered in conjunction with experiments demonstrating that zoanthid weight loss during starvation in the light is less extensive than weight loss in the dark (Sebens, 1977), we conclude that zoanthids derive substantial benefit from translocated algal photosynthate and that this benefit may vary in a species-specific manner.

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LITERATURE CITED

KOEHL, M. A. R. 1977. Water flow and the morphology of zoanthid colonies. *Proc. Third Int. Coral Reef Symp.*, Miami, FL **I**: 437–444.

LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, AND R. J. RANDALL. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**: 295–275.

MCCLOSKEY, L. R., AND L. MUSCATINE. 1984. Production and respiration in the Red Sea coral *Stylophora pistillata* as a function of depth. *Proc. R. Soc. Lond. B.* (In press.)

MCDUFF, R. E., AND S. W. CHISHOLM. 1982. The calculation of *in situ* growth rate of phytoplankton populations from fractions of cells undergoing mitosis: a clarification. *Limnol. Oceanogr.* **27**: 783–788.

MUSCATINE, L. 1980. Productivity of zooxanthellae. Pp. 381–402 in *Primary Productivity in the Sea*, P. Falkowski, ed. Plenum Press, New York.

MUSCATINE, L., L. R. MCCLOSKEY, AND R. MARIAN. 1981. Estimating the daily contribution of carbon from zooxanthellae to coral animal respiration. *Limnol. Oceanogr.* **26:** 601–611.

MUSCATINE, L., P. FALKOWSKI, AND Z. DUBINSKY. 1983. Carbon budgets in symbiotic associations. Pp. 649–659 in *Endocytobiology II: Intracellular Space as Oligogenetic Ecosystem,* H. E. A. Schenk and W. Schwemmler, eds. Walter de Gruyter, Berlin.

MUSCATINE, L., P. FALKOWSKI, J. PORTER, AND Z. DUBINSKY. 1984. Fate of photosynthetically fixed carbon in light and shade adapted colonies of the symbiotic coral *Stylophora pistillata. Proc. R. Soc. Lond. B.* (In press.)

PETERSON, B. J. 1980. Aquatic primary productivity and the ^{14}C-CO_2 method: A history of the productivity problem. *Ann. Rev. Ecol. Syst.* **11:** 359–385.

PORTER, J. W. 1976. Autotrophy, heterotrophy and resource partitioning in Caribbean reef-building corals. *Am. Nat.* **110:** 731–742.

SEBENS, K. P. 1977. Autotrophic and heterotrophic nutrition of coral reef zoanthids. *Proc. Third Int. Coral Reef Symp.* Miami, FL **I:** 397–404.

SEBENS, K. P. 1982. Intertidal distribution of zoanthids on the Caribbean coast of Panama: Effects of predation and desiccation. *Bull. Mar. Sci.* **32:** 316–335.

SMITH, R. E. H. 1982. The estimation of phytoplankton production and excretion by Carbon-14. *Mar. Biol. Lett.* **3:** 325–334.

STRATHMANN, R. R. 1967. Estimating the organic carbon content of phytoplankton from cell volume or plasma volume. *Limnol. Oceanogr.* **12:** 411–418.

STRICKLAND, J. D. H., AND T. R. PARSONS. 1977. A practical handbook of seawater analysis. *Fish Res. Board Can. Bull.* **167:** 267–279.

TRENCH, R. K. 1974. Nutritional potentials in *Zoanthus sociatus* (Coelenterata, Anthozoa). *Helgol. Wiss. Meeresunters.* **26:** 174–216.

TRENCH, R. K. 1981. Cellular and molecular interactions in symbioses between dinoflagellates and marine invertebrates. *Pure Appl. Chem.* **53:** 819–835.

VON HOLT, C., AND M. VON HOLT. 1968. Transfer of photosynthetic products from zooxanthellae to coelenterate hosts. *Comp. Biochem. Physiol.* **24:** 73–81.

WILKERSON, F. P., G. MULLER PARKER, AND L. MUSCATINE. 1983. Temporal patterns of cell division in natural populations of endosymbiotic algae. *Limnol. Oceanogr.* **28:** 1009–1014.

Reference: *Biol. Bull.* **167**: 488–494. (October, 1984)

MICROSCOPIC OBSERVATIONS OF CRYPTIC POLAR BODY PRODUCTION AND SPIRALIAN ORGANIZATION IN THE EGG OF *ILYANASSA OBSOLETA*

MARJORIE MCCANN-COLLIER

Biology Department, Saint Peter's College, Jersey City, New Jersey 07306 and Marine Biological Laboratory, Woods Hole, Massachusetts 02543

Abstract

Light microscopy of semi-thin sections and scanning electron microscopy reveal a pit at the animal pole of *Ilyanassa obsoleta* during Meiosis I. The incipient polar body is within the pit. Sections show the spindle at an oblique angle to the egg axis, an early expression of the spiralian organization of this egg. Surface changes on the incipient polar body are similar to those described by others for the polar lobe of *Nassarius reticulatus*.

Introduction

In the marine mud snail *Ilyanassa obsoleta* the egg is ripe at late prophase of Meiosis I and fertilization is internal. The eggs are fertilized in the pallial oviduct, and subsequently surrounded by a viscous fluid, enclosed in capsules (usually in groups of 50–250), and deposited on an external substratum. Normally, the eggs are deposited before maturation is complete, but there is great variability in the time elapsed between fertilization and deposition—in some cases, 24 h or more (Collier, 1981). Thus, the time of deposition is unreliable in determining the stage of development, and selecting just-laid capsules does not insure that eggs pooled from these different capsules are the same fertilization age. Collier (pers. comm.) found that a reliable criterion is the appearance of a dark spot at the animal pole. This spot, which can be seen under low magnification, is found only in the freshest eggs (preceding first polar lobe formation), lessens in intensity after 20–30 minutes, and disappears before the second polar lobe forms.

For many years this dark spot was thought to be the germinal vesicle and its disappearance, the breakdown of that body. Recently, light microscopy of semi-thin sections of this stage have shown differently: in reality the spot is a depression, or pit, at the bottom of which the first polar body is forming. Scanning electron microscopy has confirmed this observation.

Materials and Methods

Adult snails were maintained and freshly laid capsules of fertilized eggs were obtained as described by Collier (1981). Eggs were removed from the capsules and fixed either in (1) a solution of picric acid to saturation and 2% paraformaldehyde in 0.10 *M* NaH_2PO_4 buffer at pH 7.3 (PAF), which is a modification of a procedure of Stefanini *et al.* (1967), for 2 h at room temperature, rinsed in buffer, left in lithium-saturated buffer overnight, and rinsed again in buffer, or (2) in 1% OsO_4 in 0.4 *M* sodium acetate, pH 6.0, for 1 h at room temperature (Burgess, 1977). The PAF

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fixative, which was developed by Stefanini and others (1967) for electron microscopy of the acrosome and plasma membrane of mammalian sperm, as modified here proved to be the fixative of choice for *Ilyanassa* ovary and eggs. The yolk platelets, which are extremely dense and comprise 32% of the egg volume, are more readily and effectively fixed by this rapidly penetrating formula than by glutaraldehyde. For light microscopy osmium tetroxide offers no advantages and significant disadvantages *e.g.*, the density of its staining, interfere with subsequent staining, and other treatments.

For light microscopy, eggs were embedded in glycol methacrylate (Leduc and Bernhard, 1967) and sectioned at 2 μm with glass knives on a Porter-Blum ultramicrotome. Serial sections were placed on a clean glass slide, floated on water, expanded by gentle heat, and dried down. Sections were stained with azure B (Flax and Himes, 1952) and fast green FCF (Himes and Moriber, 1956) and observed using phase contrast and bright light optics.

For electron microscopy, the eggs were critical-point dried, coated with gold palladium, and viewed with an Hitachi 405A scanning electron microscope.

Results

Observations of semi-thin sections of uncleaved *Ilyanassa obsoleta* eggs revealed a vase-shaped depression, or pit, at the animal pole. In profile, the mouth and lower portion of the pit are somewhat flared, with a narrower neck in between. At or near the bottom of the pit the first polar body is emerging. (See Fig. 1.)

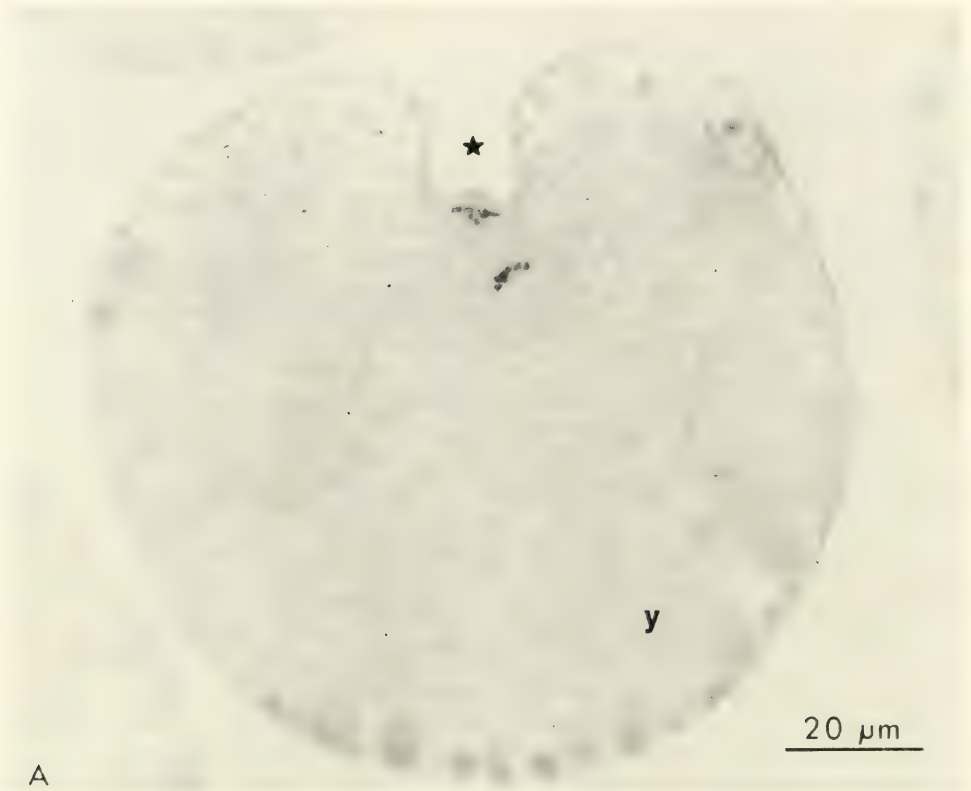
The dimensions of the pit at its greatest development, as determined from sections, are approximately 25 μm across at the mouth, narrowing to approximately 12 μm at the neck, and extending to a depth of 20–23 μm. At this phase the emerging polar body protrudes approximately 2 μm from the bottom of the pit and is thus some 20 μm from the outer surface of the egg.

In the living egg, at relatively low magnifications ($\sim$70$\times$) afforded by the dissecting microscope, the pit appears as a dark spot and cannot be discerned as an indentation, even when the egg is rolled so that the animal pole is at an angle to the optical axis. However, scanning electron microscopy of whole eggs confirmed the presence of the pit. The sides of the pit appear steep and vertically ridged and the surface of the incipient polar body is covered with reticulating folds. Clumps of an amorphous, fluffy material are evident on the surface of the egg, but not in the pit. Typically, there are two ridges that extend from the pit walls to the emerging polar body; the other sides of the polar body are more deeply demarcated. (See Fig. 2.)

The exact time of the first appearance of the pit is not known. It is certainly postovulation, as semi-thin sections of eggs in the prepallial oviduct show no depression at the animal pole. Artificial insemination experiments (unpub. obs.) provide further information on timing: the first polar lobe appears in most artificially inseminated eggs 60–90 min (24°C) postinsemination. These eggs attained the trefoil stage (*e.g.*, when the first two blastomeres and the third polar lobe are nearly divided three ways) at 240 min postinsemination, on average, and developed into normal veliger larvae.

Observation of sectioned eggs fixed coincident with the darkest spot-appearance show the chromosomes in early telophase I and with no polar lobe. Eggs with less dark spots (regressing pits) were seen to be in mid- to late telophase I, with the beginning of first polar lobe formation occuring at the end of telophase I.

From serial sections through the animal-vegetal axis during Telophase I it is evident that the axis of the meiotic figure is not coincident with the egg's axis, rather it is at an oblique angle. Figure 1.B shows a late Telophase I stage with an angle of approximately 50° between the spindle and the egg's axis.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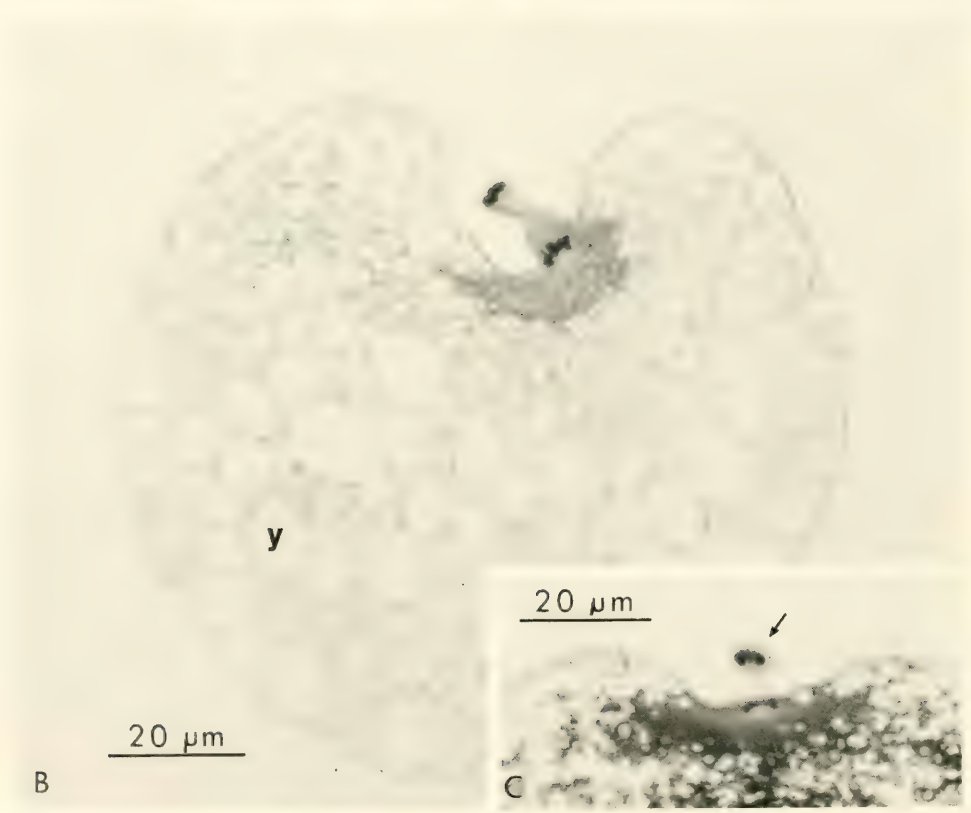
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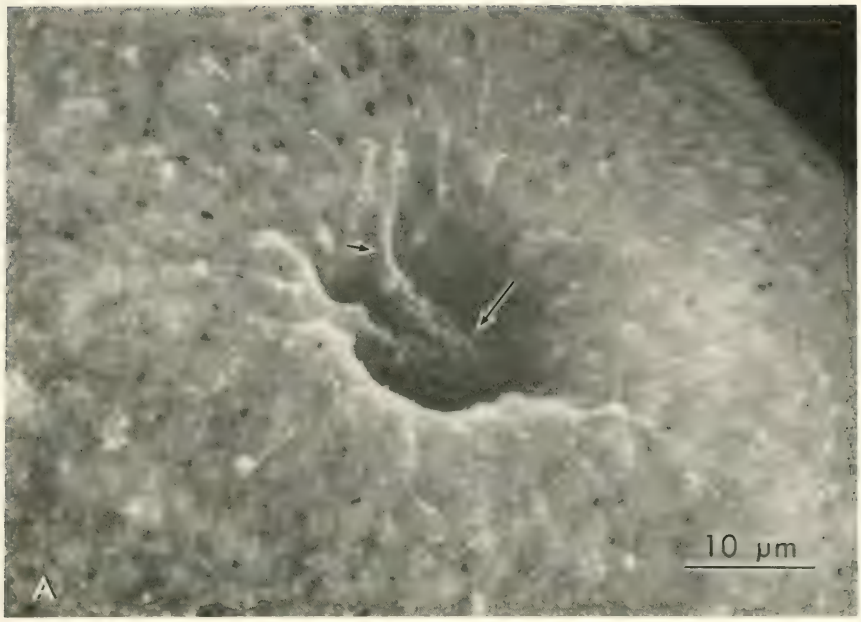

FIGURE 2. Scanning electron micrographs of Meiosis I. Fixed in picric acid-formaldehyde. A. Short arrow indicates ridge; long arrow indicates incipient polar body. B. Note reticulating folds on surface of incipient polar body.

FIGURE 1. Semi-thin sections of Meiosis I. A. Late anaphase. Star indicates pit at animal pole. Y indicates yolk platelet. Fixed in picric acid-formaldehyde; stained with azure B and fast green FCF. B. Late telophase. Fixed in picric acid-formaldehyde; stained with azure B. C. First polar body (at arrow). Fixed in OsO_4; stained with azure B.

The regressing pits show shape changes. First, the neck of the pit widens and the walls become parallel. As telophase is completed, the depression has widened at the mouth and the walls are divergent, giving a blunt conical shape to the depression; the first polar body is nearly detached and extends by a thin thread of membrane halfway, or more, the distance to the surface of the egg. At the same time, the depression has become shallower.

Sections of eggs between first and second polar lobe stages have not been examined. Sections made at second polar lobe formation show, in addition to the first polar body, telophase of Meiosis II occurring in a shallow depression of about 4 μm depth.

Discussion

Others have found alterations, in addition to those directly concerned with formation of polar bodies, in the animal pole topography of eggs. Hibbard and Wintrebert (1928) described a depression and a surrounding concavity in the animal hemisphere of *Discoglossus pictus* (Anura) eggs, which is formed when the egg is in the oviduct and lost soon after fertilization (Denis-Donini and Campanella, 1977). Wilson (1904) found that unfertilized *Dentalium* eggs frequently had a slight depression at the animal pole, just over "a very small disc of clear, dense . . . protoplasm . . ." Schmekel and Fioroni (1975), in their study of the ultrastructure of the zygote and the early segmentation stages of *Nassarius reticulatus,* included a light micrograph depicting the zygote with a slight depression, in which a polar body rests, at the animal pole. Burgess (1977) described a similar slight depression at late metaphase in *Ilyanassa obsoleta.* Judging from the size and shape of the polar lobe in his figure of a thick section and from his statement that most of the work reported was on the second meiotic apparatus, the egg appears to have been in Meiosis II. Of these examples, the *animal dimple* of *Discoglossus pictus* is most similar in form to the Meiosis I pit described here, but is approximately ten times the size of the *Ilyanassa* pit, as is the entire egg.

The surface of the emergent first polar body, with its network of folds, is different from the adjacent surface of the egg. Such a pattern of folds has been found on the surface of the polar lobe, a transient, anucleate protrusion of the vegetal pole, of *Crepidula* and *Buccinum* (Dohmen and van der Mey, 1977). The amorphous, fluffy material on the egg surface may be the remnants of a carbohydrate coat. Dohmen and van der Mey (1977) showed a carbohydrate coat on the surface of the uncleaved egg of *Nassarius reticulatus,* a close relative of *Ilyanassa obsoleta.* Taylor and Anderson (1969) reported a mucopolysaccharide component on the surface of *Ilyanassa* ovarian oocytes on the region exposed to the ovarian lumen, *i.e.,* the vegetal pole. Whether this coat is extended to the animal pole after ovulation has not been shown. In their study of the third polar lobe constriction during first cleavage of *Ilyanassa,* Conrad *et al.* (1973) showed the surface of the fully developed lobe and the two blastomeres are covered with microvilli, with no reticulating folds. To my knowledge, the first polar lobe of *Ilyanassa,* which emerges just after Meiosis I is complete, has not been examined by scanning electron microscopy. However, Dohmen and van der Mey (1977) described a circle of spirally arranged folds at the site of the incipient first polar lobe of *Nassarius reticulatus.* Dohmen and Verdonk (1979) considered two possible relations between these areas of folded surface and morphogenetic localization: they may affect the localization or activation of morphogenetic factors or they may be a reaction of the vegetal cell surface that has no primary significance for development. The observations of this surface pattern reported here for an area of the animal pole that plays no role in development suggests this is a phenomenon that can occur at either pole independently of the localization of cytoplasmic determinants.

One of the most intriguing aspects is the function of the pit. One possibility is that the area functions in fertilization. In *Discoglossus* the bottom of the dimple is the only place at which fertilization can occur (Wintrebert, 1933). Denis-Donini and Campanella (1977) found ultrastructural and biochemical features unique to the surface of the dimple bottom; they speculated that the interaction of the egg and the spermatozoan, which is nearly immotile, is a function of this specialized surface. However, in *Ilyanassa* artificial insemination of ovarian eggs, which lack a pit, is successful and produces normal embryos (author's unpub. obs.; Mirkes, 1972).

Another possibility is that the pit functions as a protective device against mechanical damage to the emerging polar body, damage which could result in cytolysis of the egg. The *Ilyanassa* egg and early developmental stages are exceptionally fragile. They do not survive cutting, injection, nor any breach of the cell membrane.

Another interesting feature of Meiosis I is that the first polar body is produced at an oblique angle to the egg axis. Spiral cleavage is usually defined in terms of the relative positions of the first four blastomeres and their successive quartets of micromeres. The term, therefore, is not applicable to the first cleavage, much less to the meiotic divisions. Conklin (1897) suggested that the first cleavage of *Crepidula,* another spiralian, be described as "*prospectively spiral and dexiotropic.*" Since the spiral pattern is the result of the obliquity of the cleavage planes, the angle of the spindle to the egg axis is a feature more useful for earlier stages, as well as more fundamental and proximate to the cause of spiral cleavage.

Wilson (1904) described the first meiotic spindle as "rotating into a radial position . . ." and presented a figure of this stage that shows the spindle at an angle of about 28° from the egg axis. In *Crepidula* the first meiotic spindle is coincident with the egg axis, but when normal cytoplasmic flows of this egg were suppressed by cold, the spindle assumed an oblique angle (Conklin, 1938). Perhaps, the cytoplasmic flows at that point normally override the basic organization that results in spiral cleavage. To paraphrase Conklin (1897), however, the direction of these spindles may be predetermined, the fact that they are determined is significant.

In my opinion, it seems reasonable that the obliquity of the meiotic spindle and the subsequent angle of extrusion of the first polar body of the *Ilyanassa* egg is an early expression of the organization that later produces spiral cleavage in this embryo.

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LITERATURE CITED

BURGESS, D. R. 1977. Ultrastructure of meiosis and polar body formation in the egg of the mud snail, *Ilyanassa obsoleta.* Pp. 569–579 in *Cell Shape and Surface Architecture,* J. P. Revel, U. Henning, and F. Fox, eds. Alan R. Liss, Inc., New York.

COLLIER, J. R. 1981. Methods of obtaining and handling eggs and embryos of the marine mud snail *Ilyanassa obsoleta.* Pp. 217–232 in *Marine Invertebrates.* Report of the Committee on Marine Invertebrates, Institute of Laboratory Animal Resources, National Research Council, National Academy Press, Washington, DC.

CONKLIN, E. G. 1897. The embryology of *Crepidula. J. Morphol.* **13:** 1–209.

CONKLIN, E. G. 1938. Disorientations of development in *Crepidula plana* produced by low temperatures. *Proc. Am. Phil. Soc.* **79:** 179–211.

CONRAD, G., D. WILLIAMS, F. TURNER, K. NEWROCK, AND R. RAFF. 1973. Microfilaments in the polar lobe constriction of fertilized eggs of *Ilyanassa obsoleta. J. Cell Biol.* **59:** 228–233.

DENIS-DONINI, S., AND C. CAMPANELLA. 1977. Ultrastructural and lectin binding changes during the formation of the animal dimple in oocytes of *Discoglossus pictus* (Anura). *Dev. Biol.* **61:** 140–152.

DOHMEN, M. R., AND J. VAN DER MEY. 1977. Local surface differentiations at the vegetal pole of the eggs of *Nassarius reticulatus, Buccinum undatum,* and *Crepidula fornicata* (Gastropoda, Prosobranchia). *Dev. Biol.* **61:** 104–113.

DOHMEN, M. R., AND N. H. VERDONK. 1979. The ultrastructure and role of the polar lobe in development of molluscs. Pp. 3–27 in *Determinants of Spatial Organization,* S. Subtelny and I. Konigsberg, eds. Academic Press, New York.

FLAX, M. H., AND M. H. HIMES. 1952. Microspectrophotometric analysis of metachromatic staining of nucleic acids. *Physiol. Zool.* **25:** 297–311.

HIBBARD, H., AND P. WINTREBERT. 1928. La formation des membranes et les changements d'aspect de l'oeuf de *Discoglossus pictus* Otth. avant la ponte. *Compt. Rendus Soc. Biol.* **98:** 1521–1523.

HIMES, M., AND L. MORIBER. 1956. A triple stain for deoxyribonucleic acid, polysaccharides, and proteins. *Stain Technol.* **31:** 67–70.

LEDUC, E. H., AND W. BERNHARD. 1967. Recent modifications of the glycol methacrylate embedding procedure. *J. Ultrastruct. Res.* **19:** 196–199.

MCCANN-COLLIER, M. 1977. An unusual cytoplasmic organelle in oocytes of *Ilyanassa obsoleta. J. Morphol.* **153:** 119–122.

MIRKES, P. E. 1972. Polysomes and protein synthesis during development of *Ilyanassa obsoleta. Exp. Cell Res.* **74:** 503–508.

SCHMEKEL, L., AND P. FIORONI. 1975. Cell differentiation during early development of *Nassarius reticulatus* L. (Gastropoda Prosobranchia). I. Zygote to 16-cell stage. *Cell Tissue Res.* **159:** 503–522.

STEFANINI, M., C. DEMARTINO, AND L. ZAMBONI. 1967. Fixation of ejaculated spermatozoa for electron microscopy. *Nature* **216:** 173–174.

TAYLOR, G. T., AND E. ANDERSON. 1969. Cytochemical and fine structural analysis of oogenesis in the gastropod, *Ilyanassa obsoleta. J. Morphol.* **129:** 211–248.

WILSON, E. B. 1904. Experimental studies on germinal localization. I. The germ-regions in the egg of *Dentalium. J. Exp. Zool.* **1:** 1–72.

WINTREBERT, P. 1933. La mecanique du developpement chez *Discoglossus pictus* Otth. de l'ovogenese a la segmentation. *Arch. Zool. Exp. Genet.* **75:** 501–539.

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PROLINE BETAINE: A UNIQUE OSMOLYTE IN AN EXTREMELY EURYHALINE OSMOCONFORMER

SIDNEY K. PIERCE[1], SAMUEL C. EDWARDS[1], PAUL H. MAZZOCCHI[2], LORI J. KLINGLER[2], AND MARY K. WARREN[1]

[1]*Department of Zoology, University of Maryland, College Park, Maryland 20742, and Marine Biological Laboratory, Woods Hole, Massachusetts 02543; and* [2]*Department of Chemistry, University of Maryland, College Park, Maryland 20742*

Abstract

The extremely euryhaline mollusc, *Elysia chlorotica,* does not utilize intracellular free amino acids for cell volume regulation during osmotic stress. Instead, *Elysia* utilizes an osmolyte previously unknown from animals, proline betaine. Although proline betaine occurs in some plants and *Elysia* forms a symbiosis with an algae, the proline betaine in *Elysia* seems to be a product of the animal.

Introduction

Two important aspects of the mechanisms utilized by cells to control intracellular water and, thereby, to survive an osmotic stress have been discovered during the past few years. First, there is mounting evidence that the cells of marine animals, traditionally held to use small molecular weight organic osmolytes such as free amino acids, have additional inorganic ionic components to the solute movements which regulate cellular volume following an osmotic stress (Pierce, 1982; Warren and Pierce, 1982; Costa and Pierce, 1983). Second, the cells of some extremely salinity tolerant invertebrate species use intracellular organic osmolytes which are quite different from the free amino acids usually encountered in less euryhaline species. For example, the cells in *Limulus polyphemus* utilize the quaternary ammonium compound glycine betaine as intracellular solute to sustain cell volumes in salinities ranging from 5% to 200% sea water (Warren and Pierce, 1982). For another example, the ascoglossan opisthobranch mollusc, *Elysia chlorotica* is the most euryhaline osmoconformer known (Pierce *et al.,* 1983b). It will survive from salinities approaching freshwater (24 mosm) up to 250% SW (2422 mosm) and is an excellent volume regulator. The intracellular amino acid pool size in *Elysia* is small, even smaller than that found in *Limulus* (Pierce *et al.,* 1983b). Thus, instead of amino acids *Elysia* utilizes a different osmolyte, but it is a molecule of unknown structure. Since the euryhalinity of *Elysia* suggests both a potential value as a model cell volume regulatory system and the possibility that the molecule may impart the extreme cellular osmotic tolerance, we have purified it from *Elysia* tissues and identified it.

Materials and Methods

Purification

Preliminary measurements indicated that the *Elysia* osmolyte could be precipitated by acid reineckate and gave a positive response to Dragendorff's reagent. The acid reineckate precipitation technique used was modified from that of Barnes and Black-

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stock (1974) and is described in detail elsewhere (Warren and Pierce, 1982). The Dragendorff's test was performed using the EtOH-NH_4OH thin layer chromatography protocol described below which is modified from Bregoff *et al.* (1953). Although not conclusive proof, these two characteristics indicated that the unknown molecule was a quaternary ammonium compound. With that as a hypothesis, we were able to purify a Dragendorff's positive substance from *Elysia* tissues using the following protocol. First, several animals were frozen on dry-ice, freeze dried, and weighed. Then, 0.5 to 1.5 gm of dry tissue were homogenized in 40% EtOH. The homogenate was brought to a boil in a water bath and then centrifuged at 20,000 $\times$ *g* for 20 min. The pellet was discarded and the supernatant shaken with ether in a separatory funnel. The ether and aqueous phases were allowed to separate and the aqueous phase collected and freeze dried. The residue was dissolved in distilled water and applied to a mixed bed ion-exchange column of Dowex-1 and Amberlite-50 (2:1). The column was eluted with five volumes of distilled water and the eluate collected as a single fraction. The fraction was freeze dried, dissolved in a small amount of 70% EtOH, and streaked across the bottom of a cellulose TLC plate. The plate was developed in EtOH-NH_4OH (8.5:1.5), dried, and the edges sprayed with Dragendorff's reagent. The TLC plate was scraped between the Dragendorff positive spots and the removed cellulose then extracted with distilled water and centrifuged at 20,000 $\times$ *g* for 20 min. The supernatant was freeze dried, the residue dissolved in EtOH and again streaked on a TLC plate. This plate was developed in BuOH, HAC, H_2O (10:3:7) dried, and sprayed with Dragendorff's reagent as before. Again, the plate was scraped, the removed cellulose extracted with water and then centrifuged at 20,000 $\times$ *g* for 20 min. Finally the supernatant was freeze dried. This purification procedure produced a brown freeze-dried residue which had a consistency of hard caramel and was extremely hygroscopic.

Identification and quantification

The residue from the purification was subjected to a variety of analytical procedures: proton and carbon nuclear magnetic resonances (NMR), UV and IR spectra. The results of these analyses suggested that the substance we purified from *Elysia* was N,N-dimethyl proline perhaps better known as proline betaine or stachydrine. The identification was confirmed by comparing the ^{1}H-NMR spectra, the high performance liquid chromatography (HPLC), and the thin-layer chromatograms of the purified material with synthetic proline betaine which had been taken through our purification protocol. Proline betaine was synthesized by heating proline and methyl iodide according to the method of Pettigrew and Smith (1977). Proline betaine was quantified by an HPLC technique (Altex Model 334 equipped with a Gilson Holochrome variable wavelength detector). The analytical column was 10 cm ODS with 3 μM diameter particles (Microsorb-short one, Rainin Instruments). Proline betaine was separated using 0.0125 *M* NaH_2PO_3 (pH 4.95) as the mobile phase and was detected at 200 n*M*. We also had success with a C_6 20 cm column (Spherisorb, Chromanetics, Inc.), but the analytical time is longer with this longer column.

Using HPLC, we have measured the amount of proline betaine in whole animal homogenates of *Elysia* adapted to salinities ranging from 87 mosm to 1834 mosm for two weeks.

Results and Discussion

The NMR spectra of the native material (Fig. 1) suggested that the *Elysia* osmolyte was proline betaine and, indeed, in all of our tests including H-NMR the native

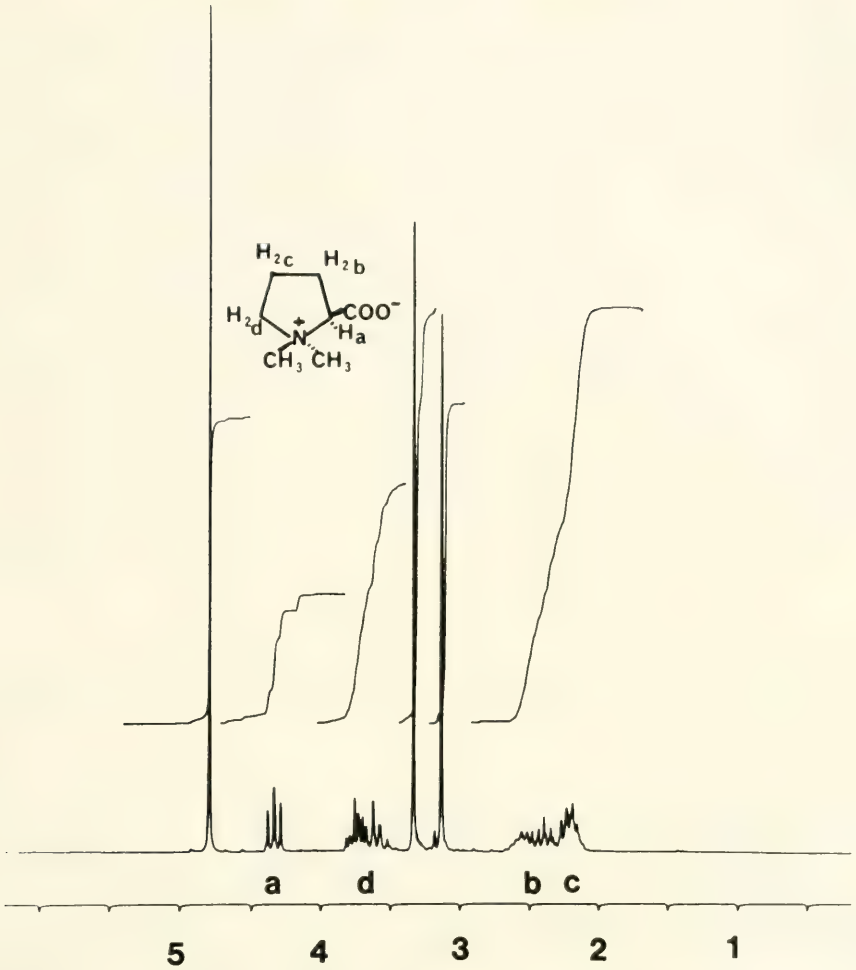

FIGURE 1. NMR spectrum of the native proline betaine purified from *Elysia chlorotica*. The letters under the peaks indicate the position in the spectrum of the correspondingly labeled protons in the structural diagram. The numbers indicate chemical shifts in ppm with respect to the external standard, sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS). The proline betaine was dissolved in D_2O and the peak at 4.8 corresponds to HOD. Spectra were recorded at 22° on an IBM WP 200 MHz NMR spectrometer.

material was identical to the authentic standard. Therefore, the molecule we extracted and purified from *Elysia* is proline betaine, contrary to an earlier report (Pierce *et al.*, 1983a).

Proline betaine concentrations were 613 μmole/g dry wt in the tissues of *Elysia* adapted to full strength sea water (926 mosm). Furthermore, the concentration of proline betaine varied with salinity ranging from 190 μmoles/g dry wt in 87 mosm to 1041 μmoles/g dry wt in 1834 mosm, the lowest and highest salinities tested (Fig. 2). Therefore, the concentration of proline betaine is of an appropriate magnitude to be the major intracellular osmolyte and its behavior with salinity adaptation indicates utilization of proline betaine for cell volume regulation. Indeed, we have shown elsewhere (Rowland and Pierce, 1984) that a pulse of proline betaine appears in the

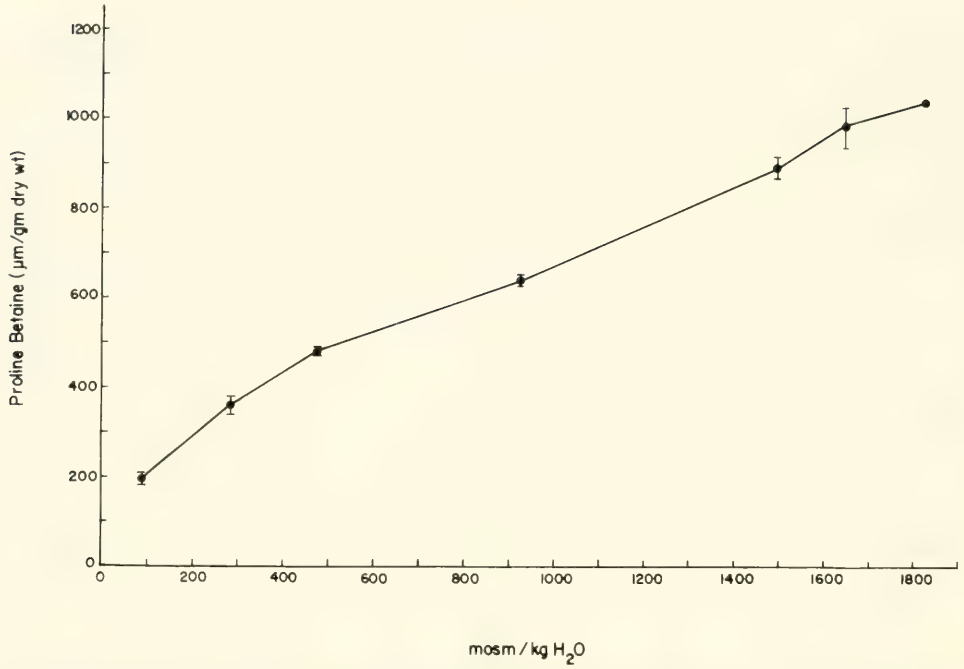

FIGURE 2. Concentrations of proline betaine in *Elysia chlorotica* adapted to various salinities for 14 days. Each point is a mean from five animals. Error bars indicate ±S.E.M.

blood of low salinity stressed *Elysia,* indicating a release of the molecule from intracellular stores in response to the stress.

Proline betaine is virtually unknown from animals, so at present nothing is known with regard to its biochemistry. Trace amounts of proline betaine have been tentatively identified by paper chromatography in the adductor muscle of *Atrina pectinata* (Hayashi and Kondsu, 1977) and by mass spectral analysis in the ovary of *Callista brevisiphonata* (Yasumoto and Shimizu, 1977). Proline betaine has been found occasionally in plants both in algae (Blunden *et al.,* 1982) and higher plants (Cornforth and Henry, 1952; Ahmad *et al.,* 1974; Sethi and Carew, 1974; Ahman and Basha, 1975) from both marine and terrestrial environments. There is almost no information on proline betaine metabolism in plants either, except that alfalfa shoots did not produce proline betaine from radioactively labelled proline (Robertson and Marion, 1959). However, *Elysia chlorotica* forms a symbiosis with the green alga *Vaucheria.* The slug seems to feed only on *Vaucheria* filaments and, as it feeds, incorporates the chloroplasts from the algae into certain cells lining the walls of the digestive tubules. The chloroplasts cause the emerald-green color of *Elysia* and also remain functional for months in the animal tissue (West *et al.,* 1984). Since proline betaine seems to be much more common to plants than animals, the possibility existed that the proline betaine in *Elysia* was arising out of the symbiosis with *Vaucheria.* An algal extract run through our HPLC protocol did not result in a peak which co-eluted with a spike of proline betaine. These results indicate that proline betaine is not present in the algae. Three additional observations also indicate that the proline betaine is produced by *Elysia* itself. First, the animals used in the salinity experiment (Fig. 2) above were not fed

during the experiment and proline betaine still increased in the animals exposed to salinities above control. Second, the *Elysia* brain which lacks chloroplasts still contains the high amounts of proline betaine. Third, *Elysia* which have been reared from eggs in the laboratory and kept in incubators for months and fed only small amounts of *Vaucheria* have the same tissue concentrations of proline betaine as freshly collected field animals (595 ± 46.0 μmoles/g dry wt and 613 ± 9.3 μmoles/g dry wt, respectively). We cannot at present conclusively eliminate the symbiotic chloroplast as the source of proline betaine, but our observations do rule out the plant as a source, and the osmolyte is an unlikely molecule for synthesis by a chloroplast.

Finally, it is unclear at present why *Elysia* should use such a unique compound as proline betaine. There is some botanical evidence that glycine betaine somehow protects some enzymes from salt inhibition (Pollard and Wyn Jones, 1979). Further in their review, Yancey *et al.* (1982) suggested that methylamines are useful osmolytes as a result of their counteraction of the protein perturbation effects of high salts or urea concentrations. *Elysia* does not accumulate urea so that possibility is unlikely. However, the possibility that, in addition to a role as an osmolyte, proline betaine may be useful as a protector of intracellular proteins from the effects of whatever intracellular salt concentrations are reached in 200% sea water seems worth testing in *Elysia*.

ACKNOWLEDGMENTS

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LITERATURE CITED

AHMAD, V. U., A. BASHA, AND ATTA-UR-RAHMAN. 1974. Structure and C-13 NMR spectrum of cadabine. *Pak. J. Sci. Ind. Res.* **17:** 109–110.

AHMAN, V. U., AND A. BASHA. 1975. Identification and C-13 N.M.R. spectrum of stachydrine from *Cadaba fruticosa*. *Phytochemistry* **14:** 292–293.

BLUNDEN, G., S. M. GORDON, W. F. H. MCCLEAN, AND M. D. GUIRY. 1982. The distribution and possible taxonomic significance of quaternary ammonium and other Dragendorff-positive compounds in some genera of marine algae. *Bot. Mar.* **25:** 563–567.

BARNES, H., AND J. BLACKSTOCK. 1974. The separation and estimation of free amino acids, trimethylamine oxide, and betaine in tissues and body fluids of marine invertebrates. *J. Exp. Mar. Biol. Ecol.* **16:** 29–45.

BREGOFF, H. M., E. ROBERTS, AND C. C. DELWICHE. 1953. Paper chromatography of quaternary ammonium bases and related compounds. *J. Biol. Chem.* **205:** 565–574.

CORNFORTH, J. W., AND A. J. HENRY. 1952. The isolation of L-stachydrine from the fruit of *Capparis tomentosa*. *J. Chem. Soc.* Part 1: 601–603.

COSTA, C. J., AND S. K. PIERCE. 1983. Volume regulation in the red coelomocytes of *Glycera dibranchiata:* an interaction of amino acid and K^+ effluxes. *J. Comp. Physiol.* **151B:** 133–144.

COSTA, C. J., S. K. PIERCE, AND M. K. WARREN. 1980. The intracellular mechanism of salinity tolerance in polychaetes: volume regulation by isolated *Glycera dibranchiata* red coelomocytes. *Biol. Bull.* **159:** 626–655.

HAYASHI, T., AND S. KONOSU. 1977. Quaternary ammonium bases in the adductor muscle of fan-mussel. *Bull. Jpn. Soc. Sci. Fish.* **43:** 343–348.

PETTIGREW, G. W., AND G. M. SMITH. 1977. Novel N-terminal protein blocking group identified as dimethylproline. *Nature* **265:** 661–662.

PIERCE, S. K. 1982. Invertebrate cell volume control mechanisms: a coordinated use of intracellular amino acids and inorganic ions as osmotic solute. *Biol. Bull.* **163:** 405–419.

PIERCE, S. K., P. H. MAZZOCCHI, L. J. KLINGLER, AND S. C. EDWARDS. 1983a. *Elysia* uses a novel molecule for cell volume regulation. *Am. Zool.* **23:** 994.

PIERCE, S. K., M. K. WARREN, AND H. H. WEST. 1983b. Non-amino acid mediated volume regulation in an extreme osmoconformer. *Physiol. Zool.* **56:** 445–454.

POLLARD, A., AND R. G. WYN JONES. 1979. Enzyme activities in concentrated solutions of glycine betaine and other solutes. *Planta* **144:** 291–298.

ROBERTSON, A. V., AND L. MARION. 1959. The biogenesis of alkaloids. XXIII. The role of proline and ornithine in the biogenesis of stachydrine. *Can. J. Chem.* **37:** 1197–1200.

ROWLAND, L. M., AND S. K. PIERCE. 1984. The utilization and fate of quaternary ammonium compounds during low salinity adaptation in *Elysia chlorotica*. *Physiol. Zool.* (In press.)

SETHI, J. K., AND D. P. CAREW. 1974. Growth and betaine formation in *Medicago sativa* tissue cultures. *Phytochemistry* **13:** 321–324.

WARREN, M. K., AND S. K. PIERCE. 1982. Two cell volume regulatory systems in the *Limulus* myocardium: an interaction of ions and quaternary ammonium compounds. *Biol. Bull.* **163:** 504–516.

WEST, H. H., J. HARRIGAN, AND S. K. PIERCE. 1984. Hybridization of two populations of a marine opisthobranch with different developmental patterns. *Veliger* **26:** 199–206.

YANCEY, P. H., M. E. CLARK, S. C. HAND, R. D. BOWLUS, AND G. N. SOMERO. 1982. Living with water stress: evolution of osmolyte systems. *Science* **217:** 1214–1222.

YASUMOTO, T., AND N. SHIMIZU. 1977. Identification of ∂-valerobetaine and other betaines in the ovary of a shellfish *Callista brevishiphonata*. *Bull. Jpn. Soc. Sci. Fish.* **43:** 201–206.

Reference: *Biol. Bull.* **167**: 501–541. (October, 1984)

ABSTRACTS OF PAPERS PRESENTED AT THE GENERAL SCIENTIFIC MEETINGS OF THE MARINE BIOLOGICAL LABORATORY AUGUST 20–23, 1984

Abstracts are arranged alphabetically by first author within the following categories: cell motility; ecology; fertilization and development; neurobiology; parasitology and pathology; and physiology. Author and subject references will be found in the regular volume index in the December issue.

CELL MOTILITY

Microtubule assembly and pronuclear movement analyzed by microbeam inactivation of Colcemid. R. E. AKINS, D. A. LUTZ, AND S. INOUÉ (Marine Biological Laboratory).

In micromolar concentrations, Colcemid (CLM) blocks tubulin polymerization *in vitro*, depolymerizes spindle microtubules *in vivo*, and inhibits movement of male and female pronuclei by preventing the growth of sperm aster microtubules. These effects are all reversed by photo-conversion of CLM to lumi-CLM by 366 nm irradiation (Aronson and Inoué 1970, *J. Cell Biol.* **45**: 470–477).

Using 366 nm microbeam irradiation of CLM-treated zygotes, we explored the conditions of tubulin availability needed to reactivate pronuclear migration. *Lytechinus variegatus* eggs were treated with 0.5 μM CLM for 30 min before fertilization, eggs were fertilized, and male pronuclei were allowed to form. Masks introduced into the field plane of a transmitted or incident illuminating microscope shaped a focused 365 ± 30 nm microbeam in the image plane. Minimal irradiation times for reversals were determined empirically from the appearance of visible sperm astral rays extending into the cytoplasm. As these rays grew to a length sufficient to reach the female pronucleus, she moved into the center of the aster and met the male pronucleus. The two then migrated to the center of the cell.

Surprisingly, the sperm aster forms and the pronuclei migrate as long as two conditions are met: (1) the small volume of cytoplasm immediately surrounding the male pronucleus is irradiated, and (2) an area of cytoplasm covering about 25% of the optical section is irradiated. It is not necessary to irradiate the female pronucleus nor the intervening cytoplasm to reactivate pronuclear migration!

We conclude that: (1) CLM must be removed from the sperm aster organizing center for sperm aster microtubules to form; (2) tubulin released from CLM during irradiation is free to diffuse through the cell; (3) this free tubulin competes favorably with CLM-bound tubulin in polymerizing onto sperm aster microtubule ends; and (4) astral ray microtubules must reach and attach to a CLM insensitive site on the female pronucleus for it to migrate.

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Steady currents go through Acetabularia crenulata: *a vibrating probe analysis.* ELIZABETH A. BOWLES (Wake Forest University) AND NINA STRÖMGREN ALLEN.

Large, steady light responsive currents go through the giant, marine unicellular alga, *Acetabularia crenulata*. These currents were measured with the vibrating probe system (Jaffe and Nucitelli 1974, *J. Cell Biol.* **63**: 614–628) at the National Vibrating Probe Facility in Woods Hole, Massachusetts. The vibrating probe is a nonintrusive platinum black tipped electrode that measures current density by detecting external voltage differences at the two extremes of its vibrational excursion through a conducting medium. Currents of *Acetabularia* cells grown at 27°C in Schreiber solution in a 8:16 hour light:dark regime were measured at noon and midnight, the midpoints of each light and dark period. When, at noon, *A. crenulata* cells are illuminated with actinic light (intensity: 300–500 $\mu W\ cm^{-2}$) large currents, with current densities (δ) ranging from 10–500 $\mu A\ cm^{-2}$, enter the rhizoid. Smaller currents (δ = 1–20 $\mu A\ cm^{-2}$) leave the stalk along its entire length, diminishing towards the apical end. When the same cells are measured at midnight under 546 nm green light, all currents are dramatically diminished (δ = 1–5 $\mu A\ cm^{-2}$), except for an occasional single large localized, inward current at a point on the rhizoid. Rough calculations indicate that the amounts of inward and outward currents were approximately equal for both white and green light trials.

The observed currents are real currents, not just a cellular response caused by a stirring effect created by the probe, since with an increased amplitude of vibration there was a proportional increase in the pen displacement.

These currents are light responsive. If during daylight hours, the lights are extinguished, the currents decay within two minutes to values near or at zero. The currents do not recover immediately when the cell is again exposed to white light; a recovery period usually lasts 10–20 minutes, and often the currents return to values slightly less than their original. If, during night time measurements, the cell is exposed to white light, the currents rise dramatically. Increasing light intensities cause increased currents; maximal currents are produced when the light intensity falls between 300 and 700 $\mu W\ cm^{-2}$.

Brain-spectrin (fodrin) distribution in the squid giant axon. KARL R. FATH AND RAYMOND J. LASEK (Case Western Reserve University).

The axoplasm of the squid (*Loligo pealei*) can be extruded from its plasma membrane leaving a 5–10 μm cortical rim of axoplasm with the glial sheath. This extrusion occurs at a sharp boundary with no apparent mixing of materials between the cortical and central axoplasm. This physical separation allows biochemical analysis of the proteins found in each compartment. Axoplasmic spectrin (fodrin) was identified by SDS polyacrylamide gel electrophoresis (PAGE) as a 235/240 kilodalton (kD) doublet. It has a similar spot pattern on two-dimensional PAGE as mammalian neuronal spectrin. Furthermore the 240 kD band was specifically stained with a fodrin antibody (Levine 1981, *J. Cell Biol.* **90:** 631–643) on Western blots. The central portion of the axon contains 80% of the total spectrin as shown by quantitative densitometry of one-dimensional PAGE. Preliminary experiments in which the glial cells were stripped from the axon suggest the remaining 20% in the sheath fraction is localized in the cortical axoplasm. Since this region contains only 10% of the total axonal volume, we calculate that spectrin is 2–3 fold more concentrated here than in the central axoplasm.

We conclude that although spectrin is concentrated in the cortical axoplasm the vast majority is found in the interior of the axon. This is rather surprising because previous light microscopic studies suggest that spectrin is almost exclusively found cortically in other neurons. We propose that axonal spectrin may have an important role cross-linking members of the internal cytoskeleton in addition to any role at the plasma membrane.

An electrophoretic model of axonal organelle transport. LIONEL F. JAFFE (Marine Biological Laboratory).

Sheetz *et al.* (1984, *J. Cell Biol.* in press) present evidence that a protomotive force somehow drives fast axonal transport in squid axoplasm. Their experiments were suggested by studies of spinning bacterial flagellae, and were initially interpreted by flagellar models like that of Khan and Berg (1981, *Cell* **32:** 913–919); one in which each forward step of the spinning flagellum is Brownian. Here I sketch an essentially electrophoretic model.

In this model, the organelle is attached to a microtubule by two 'rails.' Between the rails lies a proton permeable 'suborganelle channel.' The organelle pumps protons into the front end of this channel. These leak back into the organelle from this channel's rear end. The resulting channel current maintains a voltage difference, gradient, or field along it. This field in turn acts on fixed negative charges on the organelle so as to pull it forward.

This voltage difference also drives a reverse, external current loop which flows forward out of the channel's front orifice and returns (again flowing forward) through its rear opening. However, the organelle over these orifices is *not* lined by fixed charges. Therefore, this reverse current exerts no electrophoretic force on the organelle as it traverses these orifices. Rather, it mechanically drags the organelle forward. Thus both branches of the organelle current drive it forward.

The smallest version of this device would have one proton pump over the channel's front, one proton leak over its rear, and one fixed negative charge on the organelle's surface in between.

Supported by the National Vibrating Probe Facility.

Microtubule-associated proteins (MAPs) of dogfish brain and squid optic ganglia. GEORGE M. LANGFORD (University of North Carolina, Chapel Hill), ERROL WILLIAMS, AND DARRYL PETERKIN.

High molecular weight (HMW) MAPs 1 and 2 are common proteins in the neurons of the brain of all vertebrate species thus far studied. Experiments examined for these HMW/MAPs in the brains of the smooth dogfish shark (*Mustelus canis*) and the optic lobes of the squid (*Loligo pealii*). Microtubule proteins

(MTPs) were purified by a temperature dependent assembly-disassembly procedure. Microtubules were assembled at 30°C rather than at the physiological temperature for the organism because previous data have shown that at 20–22°C, HMW/MAPs are not present in the tubulin samples after two cycles of assembly-disassembly (Langford 1978, *Exp. Cell Res.* **111**: 139–151). At 30°C, HMW/MAPs co-assembled with the tubulins. Analysis of the polypeptide composition of the purified MTPs on 10% SDS polyacrylamide gels showed that dogfish brain contains two HMW proteins similar in molecular weight to MAPs 1 and 2 from cow brain. In addition to MAPs 1 and 2, there was one minor HMW protein that migrated between MAPs 1 and 2. This protein was labelled MAP I to indicate that its molecular weight is intermediate relative to MAPs 1 and 2. The dogfish HMW/MAPs represent 15–20% of the MTPs based on calculations of the % areas under the protein peaks of densitometric scans. MAP 2 of dogfish brain, like that of cow brain, was stable after boiling at 100°C for 5 min while MAP 1 was heat labile. After the 100°C heat treatment, which precipitated the tubulins, dogfish samples revealed a protein that ran as a broad band and that co-migrated with the tau proteins of cow brain. Squid MTPs, purified by the same procedure and assembled at the same temperature, contained four HMW proteins. When run on 10% gels, the two upper bands co-migrated closely with MAPs 1 and 2 of cow brain. These two proteins were therefore labelled MAPs 1 and 2. The third band from the top of the gel was presumed to be a contaminant since it was one of the proteins in very high concentration in the initial extract where the tubulin concentration was relatively low. The identity of the fourth protein was not clear but was not assumed to be a MAP since it was well below the molecular weight range of MAPs 1 and 2.

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Adenlyl imidodiphosphate (AMP-PNP) a non-hydrolyzable analogue of ATP produces a stable intermediate in the motility cycle of fast axonal transport. RAYMOND J. LASEK AND SCOTT T. BRADY (Department of Developmental Genetics and Anatomy, Case Western Reserve University, Cleveland, Ohio 44106).

Fast axonal transport may involve an ATPase-based motility cycle. To explore this possibility, we tested the effects of AMP-PNP on particle movement in axoplasm extruded from squid giant axons. Axoplasm was placed in a perfusion chamber and particle movement was observed by video enhanced contrast-differential interference microscopy. Perfusion with 0.5–5.0 m*M* AMP-PNP blocked the movement of particles. Particle movement stopped immediately on isolated fibrils at the periphery of the axoplasm. However, particles that were free in solution continued to bind to the isolated fibrils and then remained attached for more than 30 minutes. 0.5 m*M* AMP-PNP blocked particle movement in an equal concentration of ATP suggesting that the ATPase has a similar affinity for AMP-PNP and ATP. The addition of excess (10 m*M*) ATP reversed the effects of 1 m*M* AMP-PNP; and particles began moving along the fibrils within a minute of the replacement of AMP-PNP by 10 m*M* ATP. These results indicate that the force generating mechanism for particle translocation includes an ATPase. Further, the cleavage of ATP is apparently required for the release of vesicles after they bind to the force generating complex. That is, the ATP binding site on the ATPase may be coupled to the binding site for the vesicle, so that the cycle of association-dissociation between the vesicle and the force generating complex is coordinated with the tranlocation step.

Cross-bridges between axonally transported vesicles and microtubules in cold-blocked squid axoplasm. ROBERT H. MILLER AND RAYMOND J. LASEK (Department of Developmental Genetics and Anatomy, Case Western Reserve University, Cleveland, Ohio 44106).

Fast axonal transport of small vesicles and other membranous particles in axoplasm extruded from the squid giant axon can be observed by video enhanced microscopy. The vesicles appear to move in linear pathways along fibrils which may correspond to microtubules. To allow ultrastructural analysis of the association of rapidly transported vesicles with microtubles, the moving particles were trapped by focally cooling (*i.e.*, cold-blocking) axoplasm for 15–30 min. Then, the preparation was briefly rewarmed to insure that the accumulated particles were actively moving when the axoplasm was fixed. Fixation was accomplished by perfusion with a phosphate buffered isotonic 5% glutaraldehyde solution and the samples prepared for transmission electron microscopy. Transverse and longitudinal sections through regions of the axoplasm on either side of the cold-block demonstrated that large numbers of membranous particles accumulated. Whereas synaptic vesicles and mitochondria accumulate proximally, degenerating mitochondria and complex membranous particles accumulate distally. The vesicles were localized within specific domains of the axoplasm and high magnification revealed that the majority of these vesicles were attached to microtubules by cross-bridges. Transverse sections showed that a single vesicle could be attached to more than one microtubule.

Longitudinal sections showed that a single microtubule could contact a vesicle by more than one cross-bridge. Spacing of the cross-bridges on the individual microtubules was small enough so that even the smallest vesicles (30–50 nm diameter) were attached by more than a single cross-bridge. These observations are consistent with a cross-bridge model of axonal transport, in which force generating cross-bridges which attach to vesicles are located along microtubules.

Correlates of electrical and mechanical activity during ciliary reversal in the ctenophore Pleurobrachia pileus. ANTHONY G. MOSS AND SIDNEY L. TAMM (Boston University Marine Program, Marine Biological Laboratory).

The tentaculate ctenophore *Pleurobrachia pileus* undergoes a unilateral reversal in effective stroke direction of its ciliary comb plates during prey capture and feeding. Five Hz electrical stimulation via suction electrode of a tentacle or the body surface of immobilized animals elicits the complete feeding sequence of mouth bending, high-speed normal beat, ciliary stoppage (including a pronounced pre-reversal curvature of the plates), and unilateral ciliary reversal on the stimulated side. Preparations for intracellular recording which consist of a longitudinally split subtentacular comb row and some surrounding ectoderm display the same sequence of ciliary motor responses upon stimulation. Recordings from the ciliated epithelial cells of the comb plate (polster cells) typically reveal a −50–−75 mV resting potential. Stimulation evokes facilitating synaptic potentials which sum to produce a slow decrease in the resting potential, while EPSPs 10–15 mV over baseline produce graded action potentials with an overshoot as large as +5 to +10 mV. Correlative analysis of this response via high-speed video microscopy shows that the action potentials are always associated with and precede pre-reversal curvature and reversed beating, which occurs at 20 Hz, as in intact animals. The slow depolarization decays over several seconds after cessation of stimulation, and may be involved in the increased rate of normal ciliary beating, which decreases with the slow repolarization. The 25–30 ms action potential evoked by stimulation via suction electrode, depolarizing current injection, or anode break excitation has the same shape regardless of stimulation method, and displays a clear after-hyperpolarization and downward inflection on the downstroke. Microsurgical removal of the ~1.0 mm long comb plate cilia eliminates the regenerative response, while leaving the synaptic responses intact but enhanced, possibly as a result of increased cell resistance. Ca^{++}-free ASW reversibly abolishes spikes elicited by current injection, while injection of EGTA (.67 *M* in histidine-buffered KCl) increases excitability and eliminates the after-hyperpolarization, leaving a plateau which lengthens the spike to 75–250 ms. Preliminary results suggest that 10 m*M* Cd^{++} and Co^{++} have no effect on the spike. Twenty m*M* TEA in artificial sea water prevents the after-hyperpolarization, increases spike height (+25–+30 mV overshooting) and length to ~100 ms. These results suggest that *Pleurobrachia* ciliary comb plates have a calcium action potential which is repolarized at least in part by a Ca^{++}-dependent K^+ current, and that the voltage-dependent conductance resides solely in the cilia, as has been demonstrated in *Paramecium*.

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Fast axonal transport (FAT) of fluorescently labelled elasmobranch synaptic vesicles in isolated axoplasm from squid giant axon. TRINA A. SCHROER (University of California, San Francisco) AND SCOTT T. BRADY.

Purified cholinergic synaptic vesicles were prepared from the electric organ of *Discopyge ommata* according to Carlson, Wagner and Kelly (1978, *Biochemistry* **17**: 1188–1199) and labelled with the rhodamine dye Texas Red. Vesicles were added to extruded axoplasm from the squid giant axon. Movement of fluorescent particles was followed in video microscopy using a SIT camera and compared with endogenous particle movements as determined by video enhanced contrast DIC microscopy. The fluorescent vesicles moved in long continuous translocations at velocities comparable to endogenous squid particles of the same diameter. Movement was ATP dependent by two criteria; inhibition of movement by adenylyl-imidodiphosphate (a non-hydrolysable ATP analog) and by the enzyme apyrase, which depletes axoplasmic ATP and ADP. Under these conditions the behavior of the fluorescent vesicles was identical to that of endogenous particles. Net direction of particle movement was examined by allowing the preparations to "run" for several hours, then visualizing the distribution of fluorescence at low magnification. Preliminary observations suggest that fluorescent material accumulated at the distal end but not at the proximal end of the axoplasm. Under the same experimental conditions, 0.1 μm fluorescent latex beads that were either untreated or preincubated with bovine serum albumin did not move or accumulate at any site in the axoplasm.

FAT of both endogenous and fluorescent vesicles is inhibited by N-ethyl-maleimide (NEM). To determine if the NEM sensitive component of FAT was associated with the membranous organelles, fluorescent vesicles were preincubated in 10 m*M* NEM, pelleted, and placed into the axoplasm. The vesicles demonstrated

motility that was indistinguishable from a control population. When 10 m*M* NEM was added to the same preparation, all motility was inhibited.

Relative amounts of the major microtubule-associated proteins (MAPs) in different areas of dogfish central nervous system. ERROL WILLIAMS, GEORGE M. LANGFORD (University of North Carolina, Chapel Hill) AND DARRYL PETERKIN.

Differences in the relative amounts of the high molecular weight (HMW) MAPs 1 and 2 exist in the dendrites, axons, and cell bodies of neurons of the mammalian brain. Experiments examined for such differences in the neurons of the central nervous system (CNS) of the smooth dogfish shark (*Mustelus canis*). The CNS was divided into three sections: brain part I (the telen-, mesen-, and diencephalon); brain part II (rhombencephalon and corpus cerebelli); and the spinal cord (a 10–12 cm segment from the anterior most portion of the cord). The percentage of grey matter was high in brain part I, low in the spinal cord, and intermediate in brain part II. The microtubule proteins (MTPs) from the three areas of the CNS were purified by a temperature dependent assembly-disassembly procedure. The protein samples were analyzed on 10% SDS polyacrylamide gels. For MTP samples obtained after the second and third cycles of assembly-disassembly, MAP 1 was present in much greater concentration than MAP 2. Densitometric scans of gels of the MTPs in the first cycled, microtubule pellet, however, revealed that the amount of MAP 1 was not greater than MAP 2 except in the spinal cord. In brain part I the amount of MAP 2 was almost twice that of MAP 1; a situation that was predicted since the percentage of grey matter in brain part I was so high. Furthermore, the relative amounts of MAPs 1 and 2 in brain part II were roughly equivalent, thereby paralleling the similar concentrations of white and grey matter in that region. After depolymerization of the microtubules in the first cycled pellet, and following centrifugation, the amount of MAP 1 was high in the first cold supernatant, relative to that of MAP 2, in all three areas of the CNS. Conversely, the results for the first cold pellet revealed that MAP 2 was enriched. These results showed that MAP 2 was fractionated away from the MTPs during the 0°C incubation and therefore the first cycled, microtubule pellet is presumed to be more representative of the relative concentrations of MAPs 1 and 2 in the tissues. Further study of the segregations of MAPs 1 and 2 between the supernatant and pellet fractions of tubulin after cold induced depolymerization may help our understanding of the way in which MAP distribution is controlled in cells.

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ECOLOGY

A preliminary study of the effect of mechanical disturbance on marine sediments. AGUSTIN LOBO ALEU. (Departament d'Ecologia, Universitat de Barcelona, Spain).

This study examined some of the variables of probable importance in the assessment of the impact of trawling.

The sediment in the study site (Great Harbor, Woods Hole, Massachusetts) is a well sorted medium sand with 5% silt-clay and 0.65% organic matter. An area 5 × 10 m was divided into control and experimental sub-areas. The sediment in the experimental sub-area was removed with a rake on 24 and 31 July. Five cores (67 mm diameter and 15 cm long) were taken by SCUBA diving in each sub-area before any disturbance, three days after the first disturbance, and 8 days after the second.

The percentage of organic matter and silt-clay did not vary between the disturbed area and the undisturbed control. Water content significantly increased in the experimental cores at 2–4 cm depth. In the disturbed area, redox potential values were lower than in the control between 0 and 4 cm. The redox discontinuity layer was between 1 and 2 cm in the control area and between 0 and 1 cm in the disturbed area.

At the end of the experiment, the control ammonia concentration was significantly different at the surface (75.4 μmols/l) from the 1 cm layer (128.1). In equivalent experimental cores there was no difference (102.7 and 107.2). Total ammonia was not statistically different (337 and 362 μmols.).

Chlorophyll *a* was statistically lower in the disturbed area (29.6 mg. Chl.a/m) than in the control (15.8) a week after the first disturbance.

Photographs taken seven days after the first disturbance show a decrease in biotic activity of the macrofauna and a color change in the sediment.

I am grateful for support from the MBL Marine Ecology course.

Coincidence and association of caulobacters and diatoms. KAREN L. ANDERSON AND JEANNE S. POINDEXTER (Public Health Research Institute of New York City).

Aerobic, heterotrophic, stalked bacteria of the genus *Caulobacter* are commonly found in freshwater and marine samples in which diatoms are abundant, often attached to the diatoms' frustules. In freshwater, both groups of organisms are prominent components of the microbiota particularly during periods and in regions of phosphorus limitation (Poindexter 1981, *Microbiol. Rev.* **45:** 123–179; Tilman 1984, *Curr. Persp. Microbial Ecol.* pp. 314–319). Our preliminary experiments revealed that marine diatoms, also, were especially favored in enrichment cultures prepared with extremely low levels of phosphate, but not in those prepared with low levels of nitrogen. The present studies were undertaken to determine whether caulobacters and diatoms occurred together in Great Sippewissett Marsh, and whether caulobacters exhibited a preference for diatoms as attachment substrata. Phase-contrast and scanning electron microscopical studies of samples of alga masses attached to rocks or sand in the marsh creeks revealed caulobacters among the variety of bacteria attached to red and green algae and the sheaths of *Schizonema,* a tubular diatom. Unicellular diatoms, in contrast, were generally either free of attached bacteria or supported a small population of almost exclusively stalked bacteria. In two-membered cultures of a marine caulobacter (VC13) and each of several test algae, motile caulobacters attached rapidly to each type of alga tested, resulting in 15–20 caulobacters per alga within 60 min of inoculation. The test algae included phototrophic and apochlorotic diatoms and unicellular green algae. The only population to which caulobacters attached poorly was a pleiomorphic population of *Phaeodactylum tricornutum* in which the presence of triradiate cells implied that the cells lacked their mucilaginous capsule and silicified wall (Lewin *et al.* 1958, *J. Gen. Microbiol.* **18:** 418–426). These results imply that caulobacters appear predominant among bacterial epibionts of diatoms not because of their preferential attachment, but because other bacteria are less capable of attachment to diatoms. The apparent lack of preference of caulobacters for diatoms as substrata further implies that the coincidence and association of these organisms in natural samples is determined principally by environmental (probably nutritional) factors; the question of whether their association is of benefit to either organism remains to be explored.

This work was supported in part by the Foundation for Microbiology.

Effects of drilling mud on the behavior, growth, and survival of young juvenile lobsters (Homarus americanus). DIANA E. BARSHAW AND JELLE ATEMA (Boston University Marine Program, Marine Biological Laboratory).

A long term study was conducted on the effects of J-5 drilling mud (DM) on juvenile lobsters. We constructed fourteen 'ant farm' aquaria (10 cm wide, 35 cm long, and 50 cm high), with dimensions insuring that lobster activity inside the burrow would be mostly visible. The tanks were ⅔ filled with mud collected from a local harbor and kept on a natural light:dark cycle. Ambient unfiltered sea water flowed through the tanks. A 1 mm layer of DM was spread on the surface of five tanks, and a 1 mm layer of barite-bentonite (BB) on the surface of four tanks. The remaining five tanks were mud controls. BB is the major constituent of all DMs but lacks its sometimes toxic additives. It constituted a test for the purely physical effects of DM. We introduced three 4th stage lobsters into each tank and observed, for eight months, their settling and burrowing behavior, and their activity, feeding, and molting. DM and BB tanks received a 1 mm layer of DM or BB once each month.

Lobsters in DM treatment settled significantly later and after six months suffered significantly higher mortality than lobsters in either of the other treatments (t-test, $P < 0.05$). Because early mortality in the DM treatment had caused the surviving lobsters to live alone longer than in other treatments, DM lobsters should be expected to be larger than controls. However, at the end of the experiment DM lobsters weighed less then the lobsters from the other treatments, although this was not statistically significant. Both DM and BB lobsters lacked burrows a significantly greater percent of the time than the control mud lobsters (difference in proportions test, $P < .01$).

The results showed that long term exposure to very low levels of J-5 DM had deleterious effects on juvenile lobsters, and that the physical properties of DM alone can hinder burrow construction which in the field would expose the lobsters to increased predation.

Supported in part by a NWF-API Environmental Conservation Fellowship.

Substratum hydrophobicity and larval settlement in Janua (Dexiospira) brasiliensis *Grube (Polychaeta: Spirorbidae).* J. N. P. BLACK (Harvard University).

Larvae of the spirorbid *Janua (Dexiospira) brasiliensis* Grube were obtained from adults collected off eelgrass (*Zostera marina*) in Great Harbor, Woods Hole, Massachusetts during July and August 1984. All larvae had eye spots, shell glands, and were motile. Larvae (n = 990) were exposed to two plastic (P1 and

P2) and one glass (G) surface which spanned a hydrophobicity gradient from a middle to a low value of the range of natural surfaces in the ocean. The contact angles of these surfaces were: P1 = 28.4° ± 0.14° (most hydrophobic), P2 = 23.3° ± 0.8°, and G = 18.8° ± 0.9° (least hydrophobic). Interpretation is complicated by low settlement at times. On days when settlement of >30% of the larvae occurred overall, larvae displayed a statistically significant preference for settlement on the surfaces in the order P2 > G > P1 with mean settlement for the surfaces at P2 = 48%, G = 43%, and P1 = 24%. *Janua* may differ in settlement preferences from species of marine invertebrates tested by others, or the effect of hydrophobicity may be confounded with chemical differences between substrata.

This work was supported by the MBL Marine Ecology course.

A new topoisomerase in Escherichia coli*???* SKY BLUE AND JOHN E. CRONAN, JR. (University of Illinois).

The ability of bacteriophage PR4-infected *Escherichia coli* to incorporate ^{3}H-thymine into TCA-precipitable material in the presence of nalidixic acid suggests that PR4 DNA replication does not require *E. coli* DNA gyrase. We tested this hypothesis by determining (1) the effect of other DNA gyrase inhibitors on ^{3}H-thymine incorporation, and (2) the nature of the DNA synthesized in the presence of nalidixic acid.

Incorporation of ^{3}H-thymine was measured in both uninfected and PR4-infected *E. coli*. The cultures contained either no inhibitor, nalidixic acid (which inhibits the DNA gyrase A subunit), or coumermycin (which inhibits the DNA gyrase B subunit). Nalidixic acid completely blocked ^{3}H-thymidine incorporation in uninfected cells. As previously observed, ^{3}H-thymine was incorporated in nalidixic acid-treated PR4-infected cells. In contrast, coumermycin blocked ^{3}H-thymine incorporation in both uninfected and PR4-infected cells.

DNA extracted from cells sampled during the ^{3}H-thymine incorporation experiment was hybridized to unlabeled PR4 DNA. Similarly high levels of hybridization were observed for DNA extracted from either untreated or nalidixic acid-treated PR4-infected cells. Minimal hybridization was observed with DNA from coumermycin-treated PR4-infected cells. No detectable hybridization was observed with DNA from any uninfected cells.

The similar levels of hybridization of DNA extracted from untreated and nalidixic acid-treated infection cultures indicate that phage DNA is synthesized in the presence of nalidixic acid. The inhibition of phage DNA synthesis by coumermycin, however, suggests that DNA gyrase or a related topoisomerase is required for phage PR4 DNA synthesis. PR4 may induce the synthesis of a nalidixic acid-resistant protein similar to the DNA gyrase A subunit gyrase. This protein could combine with either the host DNA gyrase B subunit or an analagous coumermycin-sensitive phage-encoded protein to produce an active PR4-specific topoisomerase.

Changes in sediment stability caused by addition of sediment-bound #2 fuel oil. DONALD R. BRYANT (Boston University Marine Program, Marine Biological Laboratory).

Benthic communities can have a great influence on the stability of sediments. Since sediment-bound oil can cause large changes in the composition of benthic communities it may be that this oil has an effect on its own transport behavior.

To test this five cores were collected from sand flats in the Little Sippewissett salt marsh four times during the summer of 1984, and individually supplied with flowing sea water in the laboratory. After four days 0.1 g/cm^2 of dried sand flat sediment containing 0, 1, 10, 100, or 1000 ppm #2 fuel oil was applied to the surface of each core. Four days later the critical velocity (u_*) of each core was measured in a 2.5 m sea water flume using the method of Nowell *et al.* (1981, *Mar. Geol.* **42:** 133–153).

There is a significant increase (ANOVA, $\alpha < .001$) in the critical velocity from .63 cm/s to 1.92 cm/s due to the addition of sediment containing 10 ppm #2 fuel oil. All other oil concentrations resulted in insignificant changes in sediment stability. Inhibition of macrofaunal bioturbation by 10 ppm oil allows an algal mat to form which binds the sediment. When oil concentrations of greater than 10 ppm are added the algae no longer grow, resulting in lower critical velocities due to the residual surface roughness caused by the now dead macrofauna.

This research was partly supported by a NWF-API Environmental Conservation Fellowship.

Isolation of fast-growing, lactate-utilizing, oligotrophic bacteria. GEOFFREY C. COMPEAU AND JEANNE S. POINDEXTER (Public Health Research Institute of New York City).

Bacteria isolated from habitats of low nutrient flux generally exhibit low maximum growth rates and an inability to initiate growth in media of high nutrient concentration. We hypothesized that these two

properties were interrelated and that both could be accounted for by one physiologic property, *viz.*, that nutrient transport was repressible by moderate, but not oligotrophic levels of substrate. To examine this hypothesis, continuous cultivation in a lactate-limited chemostat was employed to enrich organisms from water of the Great Sippewissett Marsh. The predominant organism in each steady state was isolated, and two isolates were studied in detail. Organism I had been enriched by cultivation at a flux of 1.1 μg lactate-C/ml/h and a dilution rate (D) of 0.083/h. Its exponential growth rate was 0.33/h over a 40-fold range (12.5–500 μg lactate-C/ml) of initial substrate concentration. Organism II was enriched at a flux of 4.2 μg lactate-C/ml/h and D = 0.33/h, *i.e.*, at an oligotrophic flux, but demanding a generation time of only 2 h. Its exponential growth rate was subsequently found to be 0.53/h. Both isolates grew readily in and on media with low nutrient concentrations, but only organism II was capable of initiating growth on Zobell's medium. In preliminary characterization of the transport properties of these two isolates, cells were harvested after growth in lactate-limited and lactate-excess media, and the velocity of lactate uptake was determined in 0.1 m*M* lactate. Lactate-limited cells of organism I accumulated lactate at a velocity of 0.61 nmoles/min/10^6 cells; for cells grown in excess lactate, the velocity was reduced to 0.10. Organism II, in contrast, exhibited velocities that were not significantly different for cells grown in limited and excess lactate (1.03 and 0.78 nmoles/min/10^6 cells, respectively). These results are consistent with the initial hypothesis that the low growth rate and sensitivity to high nutrient concentrations generally observed for oligotrophs may be attributable to repressibility of transport systems, since selection for a fast-growing oligotroph eliminated the sensitivity of its growth and of its transport capacity to high nutrient concentrations.

This work was supported in part by a grant from the Department of Energy.

Influence of Bittium alternatum *grazing on epiphytes of* Zostera marina (L.): *changes in epiphyte pigment content and composition.* LINDA A. FRANKLIN (Horn Point Environmental Laboratories, University of Maryland)

The effect of grazing by the gastropod *Bittium alternatum* on diatoms, and red and brown algae colonizing *Zostera marina* leaves was analyzed by surveying pigment fluorescence excitation and absorption. Four cores containing *Z. marina* were collected at 1.3 m from Great Harbor (Woods Hole, Massachusetts). Half of the plants in each core were wiped to remove large epiphytes. Artificial (polyamide) plants were placed in two pots of sediment, and all plants were left in Great Harbor for one week to allow for epiphyte colonization.

In the laboratory, cores were placed in an aquarium under artificial light. Three cores were then subjected to grazing (40 *Bittium* per core) and separated from the ungrazed controls. Plants were sampled every 4 days for 16 days. Since feeding preference tests indicated the snails preferred epiphytes on old leaves (χ^2; $P < .05$), the oldest and youngest (longest and shortest artificial) leaves were sampled. The pigment composition of leaf tips (4 cm long) was examined by excitation spectrophotometry. Excitation spectra were obtained for chlorophyll (Chl) *a* emission (680 nm ± 20 nm). No significant difference was seen between grazed and ungrazed leaves. However, epiphytes on old leaves showed a predominance of the red algal pigment, R-phycoerythrin (R-PE), while those on new leaves showed a predominance of Chl *c* and fucoxanthin, indicating the presence of diatoms and brown algae.

The effects of four factors on epiphyte pigment composition and content were compared: (1) sampling time, (2) leaf age, (3) leaf type, and (4) grazing. Epiphyte pigments were quantified by extractions of epiphytes scraped from 8 cm of leaf tissue. No significant differences in Chl *a* content were found among any of the factors (ANOVA; $P > .05$) and Chl *b* and *c* were not detected. The red algal pigment, R-PE, increased after longer colonization periods ($P < .05$), on older leaves ($P < .05$), on natural leaves compared with wiped and artificial ones ($P < .05$), and grazed leaves ($.05 < P < .10$). *Bittium* fecal pellets contained Chl *a, b, c,* C-phycocyanin, allophycocyanin, and R-PE. These data indicate that *Bittium* consumes diatoms, and red and brown algae which are epiphytic on *Zostera.* The possible increase of R-PE with grazing is interpreted as a stimulation of red algal recolonization of *Zostera marina* blades on space cleared by grazing. Rapid recolonization would ensure maximum epiphyte productivity, and consequently maximum productivity of the seagrass community.

Research was supported by the Marine Ecology course, Marine Biological Laboratory, 1984, and the Surdna Scholarship Fund.

Association between Scoloplos fragilis *(Polychaeta, Orbiniidae) and purple sulfur bacteria in Great Sippewissett Salt Marsh (Woods Hole, Massachusetts).* MARIA CRISTINA GAMBI (Laboratorio di Ecologia del Benthos, Stazione Zoologica di Napoli, Ischia, Italy).

The present work investigated possible relationships between the distribution and abundance of a sedentary polychaete *Scoloplos fragilis* (Verrill) and photosynthetic purple sulfur bacteria in Great Sippewissett Salt Marsh.

Worms and sediment were collected at four stations having both purple (with microbial layer) and clear (without microbial layer) sands. Replicate samples were analyzed for sediment grain size, and organic, water, and pigment contents.

Major differences in pigment content were recorded for the two types of sands; by contrast the other sediment characteristics were similar in all samples. The mean (three replicates) densities in 900 cm^2 of *Scoloplos fragilis* in the purple sands were always higher than in the clear sands and a significant difference (ANOVA, $P < 0.01$) was found between the two sets of samples. However, the linear regression of worm densities on pigment content was not significant. An additional, unidentified cause of variation in worm and bacteria densities may explain the observed pattern.

In laboratory experiments, pigment content was measured in fresh purple sands placed in small dishes with and without worms. After three days under illumination, an increase both in bacteriochlorophyll *a* and chlorophyll *a* was observed. This increase was higher in the worm treatments.

Three hypotheses may explain the observed association between worms and bacteria: (a) *trophic:* worms feed on the microbial layer; (b) *benefits:* worm activities and products can favor and stimulate the microbial and microalgal growth; and (c) *co-occurrence:* another physical or biological factor influences the distribution and abundance of both organisms. Only further investigations can clarify the applicability of the above considerations.

I wish to thank the MBL Marine Ecology course which supported this research.

Methanogenic bacteria isolated from the purple sand of the Sippewissett Marsh. B. R. JOHNSON AND JAMES G. FERRY (Virginia Polytechnic Institute).

Methanogenesis was observed in a superficial habitat of the Sippewissett Marsh tidal creeks in the Purple Sand layer, a 3–4 mm thick surface layer whose color is due to purple sulfur bacteria. The layer is exposed to atmospheric O_2 and oxygenated sea water with tide fluctuation. Such an environment is unlikely for methane-producing bacteria because of the close proximity to the atmosphere and the relative lack of organic matter in the underlying sand. We examined for an association between the photosynthetic purple sulfur bacteria and methanogens, in which the former might serve as a source of H_2 for CO_2 reduction by the latter. Samples of Purple Sand were inoculated into bottles containing a reduced marine mineral medium and the system was flushed with N_2/CO_2 (80/20) to remove any residual H_2 and create an anaerobic environment. Two sets of bottles were incubated, one with light and the other without; CH_4 production was observed to be far greater in bottles exposed to light, which indicated light-dependent methanogenesis. This observation was not reproducible, but the project did lead to further examination of the methanogens present. Agar roll tubes inoculated with Purple Sand enrichments in marine mineral medium yielded three distinguishable types of colonies. Microscopic examination revealed that each type was further distinguishable on the basis of cell morphology, one being a large, irregular rod that occurred in chains, a second being vibroid, and the third a slender, highly motile rod. All three types of cells exhibited fluorescence under 420 nm UV light when observed with epifluorescent microscopy, a property attributable to the F_{420} electron carrier peculiar to methanogens. In colonies located near the roll tube stoppers, cells of two of the methanogens frequently contained refractile bodies of very uniform size, one per cell, which we tentatively identify as endospores. Endosporulation is a property not previously observed in methanogens; in these isolates, its occurrence seems to be a response to O_2 diffusion into the culture.

This work was supported by a grant from the Department of Energy.

The effect of different diets on the growth of juvenile striped mullet, Mugil cephalus, *Linnaeus.* CARLOS LASTA (INIDEP, c.c. 175, 7600 Mar del Plata, Argentina) AND MIGUEL PASCUAL.

Mugil cephalus, a marine fish common in brackish water environments, is usually considered to be a detritivore. A laboratory experiment examined whether juveniles of this species (length: 33.72 ± 0.34 mm; weight: 0.41 ± 0.0067 g) can utilize detritus as a primary food.

Fish were collected from Great Sippewissett Marsh, Cape Cod, Massachusetts, in July 1984 and separated into four groups of 40 individuals each. They were fed *ad libitum* for 12 days with (1) trout chow, (2) algae (*Enteromorpha* spp.), (3) *Spartina* detritus, or (4) they were starved as a control. Fish were measured and weighed at the beginning and at the end of the experiment. Mortality was recorded daily.

Fish feeding on trout chow grew in length and weight, while those of the other diets showed no change in length and a decrease in weight.

Additional fish samples were taken from the salt marsh to estimate natural growth rate, which was 0.34 mm/day, a value comparable to the growth rate (0.36 mm/day) of fish fed with trout chow.

Condition factors ($K = (W/L^3) \times 10^5$) were calculated. For fish fed on trout chow K (1.15) was significantly higher than the initial value (1.03) and those for the other treatments (algae: 0.90; detritus: 0.83; starvation: 0.75).

Mortality for different treatments was correlated with K ($r^2 = 0.94$). This means that K can be used to estimate mortality.

Neither *Spartina* detritus, nor *Enteromorpha* spp. were adequate food for juveniles of *Mugil cephalus*. Since length of the intestine relative to body length is higher for larger fish, striped mullet that have undergone this change may be better able to use detritus or algae.

This work was carried out with financial support from the Tinker Foundation and the MBL.

The role of sediments in the distribution of Uca pugilator *and* Uca pugnax *(Crustacea, Brachyura) in a salt marsh at Woods Hole, Massachusetts.* KARIN LUNECKE (Instituto de Ecología y Evolución, Universidad Austral de Chile, Valdivia, Chile) AND EDUARDO JARAMILLO.

Fiddler crabs are generally found in intertidal soft bottom communities of the east coast of the USA, from Massachusetts to Mexico. Two species, *U. pugilator* and *U. pugnax*, are common in the Great Sippewissett Marsh. We analyzed the distribution of these species in relation to the heterogeneity of sediments present in that marsh. The analytical design included measurements of crab distribution through a 150 m transect, together with laboratory and field experiments concerning sediment selection.

U. pugilator was found through most of the transect with exception of stations with >15% of mud. *U. pugnax* was restricted to stations with high percentages of mud, organic matter, and plant cover. Laboratory sediment preference studies in 60 × 40 cm aquaria, with the substrate divided into equal sand and mud areas and using aquaria with only sand or mud as controls, showed that *U. pugilator* digs more burrows than *U. pugnax;* it digs in sand and mud, but mostly in sand. *U. pugnax* made burrows almost exclusively in mud.

Field sediment preference studies (reciprocal transplants of sand to muddy areas and *vice versa,* together with handling controls) generally agreed with the laboratory results. *U. pugilator* dug in both kinds of substrates in the sandy area but only in the sandy plots in the muddy area. *U. pugnax* made burrows only in the muddy plots, regardless of the area. The quality of sediments seems to play a more significant role in the distribution of *U. pugnax* than *U. pugilator.* For *U. pugilator,* factors other than heterogeneity of substrate, seem involved in its present pattern of distribution.

This study was carried out with financial support provided by the Tinker Foundation and the Marine Biological Laboratory.

Analysis and comparison of stomach contents of the hermit crab Pagurus longicarpus *in salt marsh sandy creek bottoms, mud flats, and rocky intertidal zones.* ABIGAIL MARGULIS (University of Rochester School of Medicine and Dentistry).

The food sources of *Pagurus longicarpus* were studied by examining the cardiac stomach contents of animals from three different habitats: sandy creek bottom, mud flat, and rocky intertidal zone. The percent composition of the stomach contents differed between habitats according to the type of sediment on which the crabs were found.

P. longicarpus were collected at the three sites and frozen immediately. Stomach contents were then removed in the laboratory, and from each sample fifty random points were assigned under a microscope to the following categories: periphytic material, sand grains, diatoms, microscopic animal parts, filamentous green algae, and miscellaneous. Hermit crab mouth parts were found in the stomachs of several animals, indicating a tendency towards cannibalism or towards feeding on dead hermit crabs. Several significant differences among the diets of *P. longicarpus* at the three study sites resulted from differences in the sediments on which the animals were found.

Specimens of *P. longicarpus* were collected, starved for four days, and then placed on sediment from their original site from which all macroscopic organic materials had been removed. After four hours, the crabs were killed and their stomach contents examined. The only significant difference between the stomach contents of these animals and of the field-fed animals was the absence of filamentous green algae from the stomachs of the laboratory-fed animals. This suggests that *P. longicarpus,* like other hermit crabs, is primarily a detrital and surface deposit feeder.

I would like to thank the MBL Marine Ecology course for financial support for this research.

Chemical communication in lobsters: information currents. DONNA MCPHIE AND JELLE ATEMA (Boston University Marine Program, Marine Biological Laboratory).

Lobsters generate a number of different water currents like many aquatic animals. With our interest in chemical communication in *Homarus americanus* we initiated a description of their anterior currents with dye visualization.

We found a strong forward current originating in two streams from the anterior gill chamber openings. The streams merge almost immediately to form one expanding cone-shaped cloud that projects forward 113 ± 28 cm equivalent to 5–6 body lengths in adults (8 cm carapace length, CL), 38.6 ± 4.4 cm or 2–3 body lengths in subadults (5.5 cm CL) and 5.8 ± 2.2 or 1–2 body lengths in juveniles (1–2 cm CL). The gill current is produced by peristaltic pumping of the scaphognatite inside the gill chamber; it draws water from below the animal entering between the walking legs.

A second current is generated by the fanning motion of the exopodites of the maxillipeds. It pulls in water from an anterior region of approximately 7 cm radius in adult animals and it completely stops and deflects the gill current. The fan current projects to about the middle of the abdomen in adults, mostly directed posteriorly along the sides of the animal but occasionally postero-laterally. Left and right sides can fan independently, resulting in modified flow patterns.

Based on the morphology of these currents we propose the following behavioral functions for them in addition to the obvious gas exchange function of the gill current. (1) Lobster body odor and urine signals can be sent in the strong forward current over much longer distances than if they were just released to diffuse in the surrounding water; this may be important in social encounters. (2) Deflection and reversal of the continuous forward current by the fanning exopodites gives the lobster control over signal transmission. (3) The fan current allows reception of anterior chemical signals; fanning is observed regularly when lobsters are stimulated.

We postulate that these two currents are used to facilitate chemical communication and thus serve as information currents.

Supported by a grant from the Whitehall Foundation (to J.A.).

Photosynthetic activity of microbial mats at Great Sippewissett Marsh. GEORGE L. MURPHY AND BEVERLY K. PIERSON (University of Puget Sound).

The effects of light intensity on $^{14}CO_2$ fixation by microbial mat populations from Great Sippewissett Marsh were examined. Three mat layers, (an upper cyanobacterial layer and two purple-sulfur bacteria (PSB) layers) were chosen for study. Each layer consisted of a mixed population, usually with one or two dominant species. Uptake in the upper cyanobacterial layer increased with increasing light intensities up to 300 W/m^2. At light intensities greater than 800 W/m^2 up to 1000 W/m^2 uptake began increasing again. The upper PSB layer, dominated by *Thiocapsa roseopersicina,* reached maximum $^{14}CO_2$ uptake at 300–400 W/m^2. The lower PSB layer, dominated by *Thiocapsa pfennigii,* reached maximum uptake at 50 W/m^2. These optimum light intensities are well above the light intensities encountered by each layer *in situ.* The green cyanobacterial layer is beneath a top layer of diatoms and cyanobacteria, and receives about 10% of total incident radiation (TIR). On a clear day this is approximately 70–100 W/m^2. The upper PSB layer receives about 1% TIR (10 W/m^2). The lower PSB layer receives about 0.02–0.05% TIR (0.2–0.5 W/m^2). Dark uptake levels were also measured. Both PSB layers showed an increase in $^{14}CO_2$ fixed with time in the dark. The cyanobacterial layer showed no dark uptake with time. Dark fixation in the lower mat layers is believed to be due to the phototrophs themselves and possibly methanogenic bacteria and other chemolithotrophs. Because optimum light intensities for $^{14}CO_2$ uptake are well above those encountered *in situ,* it is believed that other factors, including sulfide concentration, oxygen concentration, and pH account for the locations of these organisms within the mat.

Spatial relationships in the microbial mat community of Great Sippewissett Marsh using scanning electron microscopy. JO ANN M. NICHOLSON AND BEVERLY K. PIERSON (Biology Department, University of Puget Sound).

Scanning electron microscopy was used to examine the microbial community present in a laminated mat at Sippewissett Marsh. Our purpose was to determine what microorganisms are present in the five different mat layers, how depth affects community composition, whether preferential attachment of species onto sand grains occurs, and whether vertical distribution of morphological types correlates with photosynthetic pigment analysis of the different layers. Particular attention was paid to the lower levels of the mat, where photosynthetic purple and green sulfur bacteria form distinct layers, two of which (peach and lower green) have not been described before in other microbial mats.

Sand grains with their attached microbiota were collected from the middle of each layer, immediately fixed, critical-point-dried, and examined with SEM. Similar samples for TEM of thin-sectioned material were collected by John Stolz of Boston University, who examined cell ultrastructure. Population counts and cell measurements were taken from photomicrographs of assemblages typical of each layer.

The top two layers comprise filamentous cyanobacteria (*Lyngbya, Nostoc, Phormidium* and *Oscillatoria*) and diatoms. About 60% of the pink layer is made up of large semi-attached clumps of *Thiopedia* (containing gas vacuoles) and other attached smaller purple sulfur bacteria such as *Thiocapsa* spp., small *Chromatium,*

and/or *Thiocystis*. The distinctive color of the peach layer is due to *Thiocapsa pfennigii* which forms slimy layers on sand grains. *Prosthecochloris aestuarii*, a small prosthecate green sulfur bacterial species forming slimy aggregates comprises about 70% of the lower green layer beneath.

Pigment analysis (Oesterle and Pierson 1984, *Biol. Bull.* **167:** 512) supports our observations that mat layers are complex assemblages. In addition, in the lowest three layers average cell size was found to decrease with depth, and it is suggested that light penetration may somehow be involved here. The presence of gas-vacuole-containing, mat-forming microorganisms raises questions about the dynamic behavior of living microbial mats.

This study was supported in part by a grant from the Department of Energy.

Factors affecting the synthesis and release of polyphosphate in Pseudomonas vesicularis.

JERRY NICK, HILDE NISSEN, KESHAV SINGH, HARLYN O. HALVORSON (Brandeis University, Waltham, Massachusetts), AND ALEX KEYNAN.

Numerous bacteria, including those in sewage sludge, store large quantities of polyphosphate (polyP) during aerobic growth but release it under anaerobic conditions. A strain, tentatively identified as *Pseudomonas vesicularis*, was isolated from sludge by density sedimentation on Percoll gradients (ρ = 1.132 g/ml) by N. Suresh (Brandeis University). This microorganism has an unusually high polyP content equal to 31% of its total phosphate. When *P. vesicularis* is grown aerobically in Luria broth, only during the late lag phase are cells capable of degrading their polyP and changing their density (ρ = 1.01 g/ml) under anaerobic conditions. However, throughout the aerobic growth phase the cells maintained a density of 1.132 g/ml. The rate of polyP break down under the anaerobic assay conditions is increased over 5.6 fold (Δ activity/Δ mass) by the addition of 0.1 *M* inorganic phosphate (Pi) to the Luria broth. Fully induced cells break down all of their polyP within two hours. However, Pi inhibited the anaerobic polyP reaction; the K_I is between 0.1 and 0.2 *M*. Arsenate (0.1 *M*) has no effect on polyP breakdown. Ethylenediamine tetra-acetic acid (10 m*M*) slightly stimulates polyP breakdown. Transmission electron microscopy of *P. vesicularis* reveals dense granules in the log and stationary phase cells, and their absence in cells following anaerobic incubation. While the mechanism for polyP breakdown in *P. vesicularis* is unknown, these findings suggest that the enzyme(s) is induced and its activity is inhibited by Pi. Polyphosphatase, commonly thought to be the primary cause of polyP degradation, in bacteria is repressible by Pi. The failure of stationary phase cells to break down polyP under anaerobic conditions is not as yet understood.

This research was supported in part by a grant from Air Products and Chemicals Inc.

Pigment analysis of the photosynthetic bacteria of the Sippewissett Marsh microbial mats.

ADAIR L. OESTERLE AND BEVERLY K. PIERSON (Biology Department, University of Puget Sound).

The microbial mat at the Great Sippewissett Marsh is composed of five layers (in order of increasing depth: gold, green, pink, peach, and lower green) of photosynthetic microorganisms. Qualitative and quantitative studies of the photosynthetic pigments of the mat were undertaken to determine which photosynthetic pigments were present in each layer. Samples of each layer were subjected to two extraction procedures: release into aqueous medium following sonication, and extraction into methanol. The quantity of each pigment in methanol extracts was estimated from known extinction coefficients. In initial analyses, absorption spectra were determined for each layer that was visually distinguishable along a 1-cm diameter core. Subsequently, successive discs 1 mm high were separated from each core; each of the resulting samples was 79 mm^3 in volume. This procedure provided samples suitable for quantitative determination of pigment distribution with depth. Chlorophyll *a* was readily detectable in the gold and green layers, bacteriochlorophyll *a* (bchl *a*) in the pink layer, bchl *b* in the peach layer, and bchl *c* in the lower green layer. Distribution with depth was as follows: chlorophyll *a* was most abundant at 2–3 mm, bchl *a* at 4 mm, bchl *b* at 5 mm, and bchl *c* at 6–8 mm. The maximum concentration of chlorophyll *a* (65–125 μg/ml mat volume) was higher than the maxima of bchl *a* (35–55 μg/ml) and of bchl *c* (25–55 μg/ml). The concentration of bchl *b* could not be estimated due to the unavailability as yet of an extinction coefficient for this pigment. Although each pigment was distributed through more than one 1-mm region, the order of their maxima relative to depth was the same in each mat core analyzed. Since each of these pigments is characteristic of a particular type of photosynthetic microorganism, the vertical succession of the pigments implies that the bacterial community is stratified. An associated microscopical study revealed that the pigment maxima were correlated with the distribution of the several distinguishable microbial types of the mat community (Nicholson and Pierson 1984, *Biol. Bull.* **167:** 511).

This study was supported in part by a grant from the Department of Energy.

The effect of colony density on larval recruitment in two species of Bugula *(Ectoprocta) in Eel Pond, Woods Hole, Massachusetts.* MARK E. PATZKOWSKY (Department of Geology, Indiana University).

The effect of colony density on larval recruitment in *Bugula turrita* and *B. stolonifera* was studied using five fouling panels (12 × 18 cm) submerged below a floating dock. On panels previously exposed to settlement for two weeks, all fouling organisms except *Bugula* were removed from three randomly placed quadrats (3 × 4 cm). In these, *Bugula* density was manipulated, so that one quadrat contained no colonies, a second had a low density of 1–2 erect colonies/cm^2, and the third retained the extant density of 5–7 erect colonies/cm^2. Recruits of the two *Bugula* species to each area were counted and removed every 2 days for a 10-day period.

The numbers of *B. turrita* larvae settling (overall mean = 19) in different density treatments were not statistically distinguishable, even when blocked for variation between days (ANOVA; $P > .05$). For *B. stolonifera* larval settlement was the following: high = 26.9 ± 3.18, low = 24.0 ± 2.00, and no colonies = 15.9 ± 1.86. ANOVA indicates a highly significant difference ($P < .001$) in numbers of larvae settled in the high and low density treatments compared to the cleared areas. Thus *B. turrita* shows no larval habitat selection with respect to density, but *B. stolonifera* shows a marked density effect. These results demonstrate that larval habitat preference may differ markedly even among congeners.

I extend my appreciation to Chris Reed who aided in species identification. This study was supported by the MBL Marine Ecology course.

Chaetopterus variopedatus *(Renier) (Annelida: Polychaeta: Chaetopteridae): a species complex. What species are being used at MBL?* MARY E. PETERSEN (Zoological Museum, University of Copenhagen).

Chaetopterus Cuvier is a tubicolous polycheate well known to most biologists. Although *c.* 25 species have been described, the genus is currently considered monotypic, with one highly variable cosmopolitan species, *C. variopedatus* (Renier), originally described from the Adriatic.

Work in progress reveals that the genus is not monotypic and that *c.* 10 species occur in the North Atlantic and adjacent waters alone. These are ecologically and morphologically separable into two groups: larger, infaunal species with U-shaped tubes partly buried in the substrate and smaller, epifaunal species with irregular tubes affixed to hard objects.

A convenient source of gametes for studies on fertilization and development, *Chaetopterus* has been used at the Marine Biological Laboratory in Woods Hole for nearly a century. What appear to be two species from the area are present in material in the USNM, Smithsonian Institution. One has not been collected recently and may be undescribed; the other is presently common in Quissett Harbor and may be *C. pergamentaceus* Cuvier, originally described from the Antilles. Both species are infaunal, as is *C. variopedatus,* which is not cosmopolitan and perhaps not present in the western Atlantic. As *C. "variopedatus"* is an experimental animal used throughout the world, this has far-reaching implications.

Reports of work on "well known" species commonly lack adequate locality data, and voucher specimens are not often kept; this may make subsequent reidentification and comparison with other work impossible. Investigators are urged to publish complete locality data, be critical of and indicate excessive variation in experimental animals, and deposit voucher specimens in a museum.

Sincere thanks are extended to L. Bush, Gray Museum, MBL, for use of the Tiffney Laboratory; to MBL investigators and personnel for donations of *Chaetopterus;* and to E. Prosser Armstrong, R. A. Armstrong, and L. Bush for much generous support.

Littorina littorea, Arbacia punctulata *and* Strongylocentrotus drobachiensis *as grazers on* Codium fragile. MARK PEZZANO (William Paterson College of New Jersey).

The food preferences of these three grazers were examined with respect to the green algae *Ulva lactuca* and *Codium fragile,* a brown alga *Fucus vesiculosus,* and the eelgrass *Zostera marina.* In four-way choice chambers, *S. drobachiensis* ate more *Codium* than any of the other plants (ANOVA, $P < .025$). Otherwise no significant preference for any of the plants was shown by the three grazers.

Caging experiments in the field estimated potential grazing on known amounts of *C. fragile* attached to rocks. After seven days *C. fragile* biomass had consistently increased in the control cages. In cages with grazers the results were highly variable. With *Strongylocentrotus,* two cages showed an increase in the amount of *Codium,* but in one case 78% (40 g) was eaten. With *Arbacia* a decrease in biomass of *Codium* from 14.5 to 100% (5–23 g) was verified. *Littorina* caused decreases in *Codium* of no more than 5% (2.2 g).

This study was supported by the MBL Marine Ecology course.

Interference of group-dependent motility with detection of chemotaxis in cytophagas. NANCY J. SCHENCK AND JEANNE S. POINDEXTER (Public Health Research Institute of New York City).

Chemotaxis, the net movement of cells in response to chemical gradients in the environment, has been detected and characterized in a variety of flagellated bacteria. However, whether gliding bacteria are capable of this response is controversial. There have been reports of chemotaxis in *Myxococcus xanthus,* but such behavior cannot be reproducibly demonstrated. It is reasonably argued that the velocity of gliding cells is so much slower than the rate of diffusion of solutes that gradients decay before a response could possibly be manifested. The present studies were undertaken to determine whether chemotaxis could be detected in faster-moving gliding bacteria (two marine and one freshwater isolates of *Cytophaga*). In two types of assays (rate of migration and growth in soft agar, and accumulation in microdroplets of soft agar containing utilizable nutrients), neither of the marine strains exhibited a clear migratory response to nutrient concentration. In microcultures prepared in depression slides, two types of motility were observed in each of the three cytophagas; neither type was affected by an initial nutrient gradient. (1) In regions where the cells moved on the cover slip bathed in a liquid phase, individual cells glided and pivoted independently of each other, and in random directions. (2) In monolayers of cells migrating between the cover slip and the agar, net translational movement was accomplished mainly by groups of 10–50 cells arranged in parallel within arrowhead-shaped swarms. Migration of the groups was rapid (several micrometers/min) within the inhabited region, but slowed considerably upon pioneering entrance into the cell-free zone. However, once a pioneer group had left the inhabited region, other groups followed in its tracks. These observations revealed that net migration was highly dependent on transient cell aggregation into gliding swarms, and that the migration of a swarm was facilitated by previous passage of another swarm through the same region; both features are presumably evidence of facilitation of gliding by slime accumulations. In addition to slowness, the marked dependence of gliding motility on the concerted migration of cells in swarms would complicate any demonstration of chemotaxis in cytophagas.

This work was supported in part by the Foundation for Microbiology.

Clumping behavior of bacteria isolated from waste water treatment sludge. KESHAV K. SINGH, WALTER S. VINCENT (University of Delaware Newark, Delaware), AND CHRISTOPHER J. GRIFFITH.

Although many reports have appeared on the nature of the attachment of bacteria to physical substrates, very little is known about the mechanism by which bacteria attach to other bacteria. In this report, three strains of bacteria were studied. *KEWA 1, 2,* and *3* were isolated from a sludge community derived from a waste water treatment plant. These strains are Gram-negative, form large clumps with themselves, and settle very rapidly from suspension. When the cells are grown in nutrient medium (1% Bacto peptone, doubling time ± 1.5 hours) they are motile rods with single polar flagellum. When grown on dilute medium (0.1% Bacto peptone, doubling time ± 2.5 hours or a chemically defined medium containing 100 ppm glucose), all these strains form an extensive capsule which fuses with other cells to form large clumps. In this stage, the cells are hydrophobic. However, in the rich medium the motile cells do not demonstrate capsule formation, and are correspondingly non-hydrophobic. The ability to form clumps is destroyed upon heating the cells for 10 min at 50°C, even when re-inoculated into dilute medium. Although the optimal growth is at pH 7.0, the clumps are not dispersed with high (10.0) or low (4.0) pH, detergents, or chelators. Emulsan, a known inhibitor of capsule formation in many bacteria, does not affect the clumping ability of these strains. Bacterial clumping, selected by the waste water treatment process, appears to be a complex set of interactions within and between strains of bacteria. Our studies reveal some of this complexity.

Supported by a grant from Air Products and Chemicals Inc., USA.

Diurnal changes in free amino acid patterns of Zostera marina L. *roots.* ROBERT D. SMITH, A. MARSHALL PREGNALL, AND RANDALL S. ALBERTE (Barnes Laboratory, The University of Chicago).

Roots of sediment-free *Zostera marina* (eelgrass) accumulate substantial quantities of the amino acids alanine (ALA) and γ-amino butyric acid (GABA) within a few hours after oxygen deprivation, while levels of glutamate (GLU) and glutamine (GLN) decline markedly. Upon re-oxygenation of the roots, GLU and GLN increase rapidly, while GABA and ALA decline. Since eelgrass grows in sediments that are both anoxic and reducing, we investigated whether roots of *in situ* plants exhibit similar changes in amino acid content due to diurnal fluctuations in photosynthesis-dependent root oxygenation (Smith *et al.* 1984, *Plant Physiol.* **74:** 1052–1058). Eelgrass plants were collected at dawn (5:00 am) and dusk (6:00 pm) from shallow

(1.3 m depth) and deep (5.5 m depth) regions of a meadow in Great Harbor, Woods Hole, Massachusetts. Roots were immediately excised and frozen in liquid nitrogen prior to extraction of amino acids with hot ethanol. Amino acids were derivatized with o-phthalaldehyde, separated, and quantified by High Performance Liquid Chromatography. Roots of deep-growing plants had greater amounts of ALA and GABA at dawn (maximum anaerobiosis) than shallow plants, while roots of shallow-growing plants had greater amounts of GLU and GLN at dusk (maximum aerobiosis) than deep plants. These results indicate that the roots of shallow plants, which experience longer daily photosynthetic periods, recover more fully from nightly hypoxia and anoxia than roots of deep-growing plants. They also demonstrate that roots of eelgrass show metabolic adjustments to diurnal periods of anoxia that may be adaptive. Lastly, the rapid increases in GLU and GLN content during aerobic periods imply that ammonium assimilation occurs chiefly during daylight hours in eelgrass.

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Preliminary studies of bioenergetic partitioning in specimens of the salt-marsh pulmonate, Melampus bidentatus.

JAY SHIRO TASHIRO (Kenyon College), CHRIS FLEMING, AND DAN DESSNER.

Melampus is found in the high littoral zones of semi-enclosed salt marshes along the Atlantic coast. A large empirical base already exists for a population of this species in the Little Sippewissett Marsh (Russell-Hunter *et al.* 1972, *Biol. Bull.* **143:** 623–656). We now report two physiological and ecological studies on *Melampus* from this population. The first is analyses of gonadal cycling over a reproductive period. Second, studies of respiration and feeding have been completed on post-reproductive individuals.

For field and laboratory studies (Summer, 1983) snails were obtained from the Little Sippewissett Marsh (120 snails, every six days) and fixed in 12% (v/v) neutral formalin in 75% sea water. Gonads were dissected out of the visceral mass and both gonads and somatic tissues were dried to constant weight. Protein analyses were performed on gonads. There was a decrease in gonad weight during oviposition. In the second to last egg-laying cycle (19–25 June), gonad protein decreased in 2- and 3-year-old snails, but at a slower rate in 2-year-olds. There appears to be some age-specificity in gonadal constituent cycling during reproduction.

Studies of respiration (see also McMahon and Russell-Hunter 1981, *Biol. Bull.* **161:** 246–269) and feeding evaluated an ingested food allocation into total assimilation, and respired and nonrespired assimilation. Animals were maintained in an environmental chamber (24 July–5 August, 1984; 20°C, 14L:10D). The mean oxygen consumption rate for 1-year-old snails was 9.36 $\mu l \cdot h^{-1}$, 10.81 $\mu l \cdot h^{-1}$ for 2-year-olds, and 29.01 $\mu l \cdot h^{-1}$ for 3-year-olds. Feeding studies employed two artificial rations. The first consisted of lettuce and brewers yeast in a Ca-alginate matrix (AF); the second, the aforementioned recipe with added protein (AFN). No apparent differences in the ingestion rates of these two foods was observed. Feeding on AF, one-year-olds ate 9.7 (S.D. $\pm$ 5.3; n = 10) $\mu g \cdot h^{-1}$, 2-year-olds ingested 28.7 (S.D. $\pm$ 19.6; n = 10) $\mu g \cdot h^{-1}$, while 3-year-olds ingested 62.2 (S.D. $\pm$ 38.1; n = 10) $\mu g \cdot h^{-1}$. Similar levels of ingestion were observed for the AFN food.

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Salt tolerance of germination of Bacillus *spores from terrestrial and marine marsh origins.*

THERESA A. TIERNAN, ALEX KEYNAN, AND HARLYN O. HALVORSON (Brandeis University, Waltham, Massachusetts).

The salt tolerance of germination of two terrestrial spore formers, *Bacillus cereus* and *Bacillus subtilis,* was compared to the salt tolerance of strains *3A10* and *Mn1* isolated from the Sippewissett Marsh. The preparation of the spore suspension and the germination requirement of the marine marsh organism has been described (Wier *et al.* 1983, *FEMS Microbiol. Lett.* **20:** 27–30). Optimum heat activation of the marine strains was 55°C. This heat activation requirement could not be replaced by high concentrations of ammonia. The effect of 3–12% NaCl concentration on germination of all four strains was investigated. The terrestrial strains were strongly inhibited by salt concentrations higher than 1%. Both marine strains were stimulated by NaCl. One strain, *3A10,* was stimulated at salt concentrations up to 7%, and the other strain, *Mn1,* was stimulated by concentrations up to 12% NaCl. KCl interfered with NaCl-induced germination of *3A10* strongly; 1% KCl reduced germination rates by 70%, while LiCl, at concentrations up to 12%, did not interfere with Na induced germination.

The tolerance of germination to high NaCl concentrations might explain the ability of the marine organism to propagate in an environment with a rapidly changing salt concentration.

Supported by a grant from The Foundation for Microbiology.

A study of spore forming bacteria isolated from the deep sea. PETER B. VANDER HORN, THERESA A. TIERNAN, ALEX KEYNAN, HOLGER JANNASCH, AND HARLYN O. HALVORSON (Brandeis University, Waltham, Massachusetts).

Although aerobic spore formers have been known to exist in the deep sea since the 1880's, little is known of their biology. We have characterized some of the growth and germination conditions of ten deep sea isolates. Seven of these isolates came from surface sediment of a vent area located at 21°N, 109°W, and 2600 m depth; three others were isolated from a core taken at 19–09°N, 65–39°W, and 5870 m depth, two from the top, and one from 60 cm deep. Although evidence exists of psychrophilic spore formers, we isolated our samples in numbers around 10^6 cfu/g at 30°C on ZoBell sea water agar. Cell and spore morphology resemble terrestrial isolates. All vegetative isolates were motile. Six isolates were highly pigmented; methanol extracts showed typical carotinoid pigments. The temperature range of optimal growth was between 30°C and 42°C. One strain demonstrated a dependence on salt for growth. Three grew on a glucose, ammonia, and phosphate minimal media. All isolates were catalase positive and two were oxidase negative. Three strains showed a surprising resistance to the antibiotic tetracycline. Three strains oxidized Mn^{++}, a process thought to be environmentally important in deposition of this mineral (Rosson and Nealson 1982, *J. Bact.* **151:** 1027–1034). Germination of two out of six strains was activated by heat shock; maximum germination occurred in ammonia, adenosine, NaCl, and HEPES buffer. Pigmentation and temperature range suggest that these strains originated from the surface, perhaps attached to particles, but it is difficult to say whether the deep sea is a dump for surface and/or marsh marine spore formers.

Comparative reproductive adaptations of grass shrimps, Palaemonetes vulgaris *and* P. pugio *in Great Sippewissett Salt Marsh.* HONG-YOUNG YAN (Department of Zoology, The University of Texas at Austin, Austin, Texas 78712).

In Great Sippewissett Salt Marsh, Massachusetts, grass shrimps, *Palaemonetes vulgaris* and *P. pugio,* were collected by dipnet on 14, 22, and 28 July 1984. One hundred sixty-two male and 106 female *P. pugio* adults were collected. Similar effort yielded a total of 270 female and 422 male adult *P. vulgaris. P. pugio* is displaced by *P. vulgaris* from deep pools with sheltering *Codium* to waters of bare muddy bottom where the former may experience higher mortality than the latter species.

P. vulgaris matures at a smaller size than *P. pugio,* as shown by a significant difference in carapace length and body length. Linear relationships between fecundity and body length indicate that for both species fecundity increases in proportion to body size; the larger *P. pugio* has higher fecundity than *P. vulgaris.*

P. pugio has significant higher egg numbers per mm carapace length and significantly higher weight per egg (or embryo) than *P. vulgaris;* these reproductive parameters are size-dependent. A size-independent reproductive parameter, the gonado-somatic-index (G.S.I.), a relative measure of reproductive effort, shows no difference in the investment of effort per clutch between the species.

The two species have evolved different reproductive strategies. *P. pugio* which matures at a larger body size than *P. vulgaris* has a size-dependent advantage, *i.e.,* higher fecundity, which compensates for the possible greater loss from predation.

I consider the reproductive adaptations of these shrimps to be the result of responses under different predation pressures, that maximize individual life-time reproductive success in the different environment of the two species.

This study was supported in part by a summer fellowship from the Marine Biological Laboratory and the Lucretia Crocker Scholarship Fund.

FERTILIZATION AND DEVELOPMENT

Low pH sodium-free sea water reversibly blocks cleavage in embryos of Arbacia punctulata. PHILIP G. ALLEN (Program in Cell and Developmental Biology, Division of Medical Sciences, Harvard University)

Previous work (see Begg *et al.* 1982, *J. Cell Biol.* **93:** 24–32) has indicated that the cortical structure of actin in the just fertilized sea urchin egg is affected by intracellular pH. To investigate whether cytoplasmic pH might have an effect on the formation of the actin structures necessary for cleavage, one-cell embryos of *Arbacia punctulata* were exposed to pH 6.5 sodium-free sea water (NaFSW). NaCl was replaced with choline Cl and $NaHCO_3$ with $KHCO_3$ to the MBL formula. This solution rapidly lowers the intracellular pH of fertilized eggs (Shen and Steinhardt 1979, *Nature* **282:** 87–89). At various times after fertilization the embryos were washed two times into pH 6.5 NaFSW at 20°C; later the embryos were washed back

into millipore-filtered sea water (MFSW). The low pH NaFSW blocks cytokinesis when applied up to 10 minutes before cleavage. The low pH NaFSW block is reversible by washing into MFSW up to 150 minutes later; such embryos develop at least through swimming blastula stage.

The effect of low pH NaFSW appears not to be on protein synthesis, since 1 m*M* emetine blocks cleavage only when applied during the first 20 minutes of a 70 minute first cell cycle. Such embryos characteristically arrest at streak stage. The effect of pH 6.5 NaFSW is not due to the low external pH alone or to the lack of sodium alone; neither pH 6.3 MFSW nor pH 8 NaFSW has an effect on the cell cycle or cleavage. The block of cleavage by pH 6.5 NaFSW is not effective if small amounts of sodium are present.

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Hydrogen peroxide: source of hypochlorite blocking fertilization in Arbacia punctulata.

FREDRIC BLUM, CATHERINE BEARCE, KRISTINA FRENKEL, AND WALTER TROLL (New York University Medical Center)

Cupric chloride and cupric diisopropyl salicylate both block fertilization of *Arbacia punctulata* at 10^{-6} *M*. This effect of copper compounds appears to depend on the presence of hydrogen peroxide (H_2O_2) since removal of H_2O_2 by catalase abolishes the effect of Cu^{++}. Cu^{++} possibly acts by mimicking the action of superoxide dismutase (SOD) which forms H_2O_2 from superoxide anions (O_2^-). The increased H_2O_2 may be responsible for inactivating sperm. We sought to confirm this mechanism by measuring the production of H_2O_2 using horse radish peroxidase oxidation of pyrogallol sulfonphthalein in the presence of Cu^{++}. Instead of increasing H_2O_2, Cu^{++} consumes H_2O_2. This disappearance implies that Cu^{++} converts H_2O_2 formed by the *Arbacia* ovum at fertilization to a more toxic product. Cu^{++} plus H_2O_2 are more active in mutagenesis and in other oxidative properties than either Cu^{++} or H_2O_2 alone. This increased activity is presumably due to formation of hydroxyradicals ($OH\cdot$). $OH\cdot$ radicals are formed by ferrous ions from H_2O_2; yet ferrous chloride has no effect on fertilization. Moreover, the $OH\cdot$ radical scavengers mannitol and thiourea failed to counteract Cu^{+} fertility effects. Our findings support the notion that hypochlorite may be responsible for the Cu^{++}-H_2O_2 action. 2-Aminotriazole, an hypochlorite scavenger, completely counter-acts the toxic action of Cu^{++} on the sea urchin fertilization. Thus, Cu^{++} may mimick myeloperoxidase rather than SOD in oxidizing Cl^- to OCl^-. Direct conversion of $CuCl_2$ to OCl^- by H_2O_2 was demonstrated by showing conversion of taurine to taurine chloramine, a characteristic reaction of hypochlorite. Taurine chloramine converts potassium iodide to I_3^- which is readily measured colorimetrically. Our demonstration that Cu^{++} ions are capable of forming hypochlorite is of interest in rationalizing a number of Cu^{++} actions. Cupric chloride, arising readily from copper, may be responsible for the well known antifertility action of this metal.*Arbacia* may use OCl^- as a method of eliminating the entry of more than one sperm. This effect may be mediated by a myeloperoxidase contained in the sperm.

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Further studies of early determination in the polyclad embryo.

BARBARA C. BOYER (Union College).

Blastomere deletions at the two-cell stage carried out through the egg shell membrane of the polyclad turbellarian *Hoploplana inguilina* showed that this spirally cleaving embryo is determined early (Boyer 1980, *Biol. Bull.* **159:** 448–449). Successful *in vitro* fertilization leads to normal development of naked eggs, and therefore blastomere separation experiments also are possible at the two-cell stage. Fifty-four experimental larvae developed from separated cells and of these, 48% were very similar to those which develop from deletion experiments, with indistinct lobe delineation. Thirty percent were essentially "lateral-half" Muller's larvae with distinct lobes (only 22% were very aberrant). For both classes combined, the proportion of eyeless (28%) to eyed (72%) larvae was statistically the same as the proportion obtained in deletion experiments—a fact which supports the conclusion that two-cell deletion and separation are nearly equivalent experimental techniques.

Deletion of one cell at the four-cell stage through the egg shell membrane produced 106 embryos which were more complete than the "half larvae" but which were underdeveloped in one of the quadrants. Seventy-three percent of these had one eye and 27% had two eyes, which is almost exactly a 3:1 ratio. Since deletion of any one of the four cells was equally likely, this ratio strongly suggests that three of the four blastomeres directly contribute to eye formation—two constitute the eye lineage itself and the third apparently induces development of two eyes. Almost all the one-eyed forms which had asymmetric ventrolateral lobe deficiencies were missing the eye on the deficient side.

These results are strong evidence that polyclad embryos have early determinative development but

with significant cell interactions occurring during cleavage, and suggest that early determination and the quartet spiral cleavage pattern are always associated and probably represent a primitive, strongly conserved evolutionary condition.

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Synthesis of heat shock proteins during development of the starfish, Asterias forbesi.

DAVID J. GOLDHAMER (The Ohio State University), WILLIAM T. CARROLL, K. DAVID BECKER, AND ANDREW P. MCMAHON.

All organisms thus far studied respond to heat stress by the rapid synthesis and accumulation of a novel set of proteins, the heat shock proteins (hsp). In addition to expression on heat shock, some of these proteins are also synthesized as normal cellular components during development.

As a first step towards the study of heat shock gene expression during starfish development, we characterized the heat shock response in oocytes and embryos of the starfish, *Asterias forbesi.* Cultures were incubated at 18°C (control), 23°C, 27°C, and 31°C for 1 h, followed by an additional hour at the respective temperatures in the presence of ^{35}S-methionine. Labelled proteins were separated by one-dimensional SDS polyacrylamide gel electrophoresis and visualized by fluorography. No heat shock response was seen in mature and immature oocytes, and 32–64 cells stage embryos; the proteins synthesized were identical at all four temperatures. However, at the blastula stage (12 h), three proteins with molecular weights of 90K, 70K, and 40K accumulated rapidly on heat shock at 27°C and 31°C. Later stages up to the early bipinnaria larva (72 h) showed a similar coordinate induction of these proteins at 27°C—the optimum temperature for elliciting a maximal response without causing extreme developmental abnormalities. Two-dimensional gel electrophoresis resolved both hsp 90 and hsp 70 into two distinct spots varying slightly in molecular weight or isoelectric point respectively. The 90K proteins were also found prior to heat shock in 6 h and 12 h embryos though accumulation on heat shock only occurred at 12 h and later. The predominant heat shock proteins, the hsp 70 proteins, were never detected prior to heat shock. We are currently investigating the regulation of the heat shock response by cell-free translation and Northern blot analysis to determine how hsp expression is controlled in starfish embryos.

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Serotonin induction of spawning and oocyte maturation in Spisula.

S. HIRAI (Tohoku University), T. KISHIMOTO, S. S. KOIDE, AND H. KANATANI.

Injection of 0.5 ml of serotonin (5-HT) at concentrations of 20 μM or greater into the gonads of *S. solidissima* and *S. sachalinensis* induced gamete shedding in both males and females. Shedding of sperm and immature oocytes was induced within 2 to 30 min after administration of the drug. These oocytes underwent maturation and developed normally after fertilization. Treatment of isolated immature oocytes with 5-HT dissolved in natural sea water at concentrations of 10–100 μM induced germinal vesicle breakdown (GVBD) followed by polar body formation. However, 5-HT-induced matured oocytes were not fertilizable. Other neurotransmitters including dopamine, acetylcholine, and γ-amino butyric acid had no effect on either gamete shedding or oocyte maturation.

The production of maturation promoting factor (MPF) in *Spisula* oocytes undergoing maturation after insemination or treatment with KCl or 5-HT was assayed by microinjecting aliquots of cytoplasm into immature oocytes of *Asterias forbesi.* The cytoplasm of the treated *Spisula* oocytes induced GVBD while that taken from immature oocytes did not. The present findings support the thesis that MPF is produced during oocyte maturation and is not species specific.

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Development of in vitro *fertilized eggs of the squid* Loligo pealei, *and techniques for dechorionation and artificial activation.*

KAREN C. KLEIN (Department of Anatomy, University of Illinois, Urbana) AND LAURINDA A. JAFFE.

To achieve 90% *in vitro* fertilization, clear mature squid eggs are collected from the oviduct of a dissected female and washed several times with well aerated millipore filtered sea water (MFSW). Spermatophores (20–30) from the spermatophore duct of a dissected male are placed in 15 ml MFSW in a syracuse dish, and sperm are discharged with slight pressure. Approximately 200 eggs are added to this sperm suspension. After 20 minutes, fertilized eggs are washed several times in MFSW to remove sperm and spermatophore casings.

In vitro fertilized embryos arrest at the early blastoderm stage if cultured in glassware or in Falcon plastic petri dishes, to which their chorions adhere. *In vitro* fertilized embryos are successfully cultured through organogenesis to chorionated hatchlings in 60 mm Falcon plastic petri dishes lined with .2% agarose (Sigma, Type II). Embryos are cultured in MFSW at 16–18°C. The MFSW is changed every two days and any arrested embryos are removed. One to fifty embryos can be cultured per dish.

Unfertilized eggs and early cleavage stage embryos, which are difficult if not impossible to dechorionate mechanically, can be chemically dechorionated with protease (Sigma Type XIV, 1 mg/ml in MFSW). Chorionated eggs or embryos are placed in the protease solution for 20 minutes and then washed several times in MFSW. Twenty minutes post-washing the chorions elevate and wrinkle and can be mechanically removed. By one hour post-washing, eggs from some batches fall free of their chorions after gently swirling the dish. Dechorionated embryos develop to about 5th cleavage around which time development arrests. Dechorionated unfertilized eggs have not yet been successfully fertilized. Both chorionated and dechorionated squid eggs can be artificially activated with 10 μg/ml A23187.

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A novel nucleotide from Spisula *regulating meiotic arrest of oocytes.* S. S. Koide (Population Council), E. Sato, M. K. Sahni, H. N. Wood, and D. G. Lynn.

Full-grown oocytes of mouse, *Spisula,* and *Chaetopterus* are arrested at the dictyate stage of meiotic prophase. Isolated mouse and *Chaetopterus* oocytes undergo spontaneous maturation while *Spisula* oocytes retain their germinal vesicles. This report describes a nucleotide with meiotic arresting activity.

The meiotic arresting nucleotide was extracted from *Spisula* muscles by homogenization in 70% ethanol. The ethanol extract was concentrated and purified by chromatography on Dowex 1-8× column. The column was eluted in sequence with 0.001, 0.01, and 0.1 *N* HCl. The fractions were pooled, neutralized, lyophilized, and assayed. The active substance was found in the 0.1 *N* HCl fraction and further purified by reversed phase HPLC. Procedures for the isolation of mouse, *Spisula,* and *Chaetopterus* oocytes and examination for germinal vesicle breakdown (GVBD) were described in previous reports (Sato and Koide 1984, *Differentiation* **26:** 59; Sano *et al.* 1979, *Dev. Growth Differ.* **21:** 457; Ikegami *et al.* 1976, *Dev. Growth Differ.* **18:** 33).

GVBD in *Spisula* oocytes can be induced by insemination (5×10^4 sperm/ml) or treatment with serotonin (5 μM) or KCl (35 mM). *Spisula* extract at concentration of 1 mg/ml blocked oocyte maturation induced with serotonin. The block was reversible. Forskolin (2.5 μM) and isobutyrylmethyl xanthine (IBMX) (12.5 μM) blocked GVBD induced with sperm or serotonin. dbcAMP at 0.1, 0.5, and 1.0 mM and dbcGMP at 1.0 and 2.0 mM blocked GVBD induced with sperm, while higher concentrations of these nucleotides were required to block serotonin-induced GVBD. However, none of the compounds tested including *Spisula* extract influenced KCl-induced GVBD. *Spisula* extract at 1 mg/ml also depressed motility of *Spisula* sperm.

Isolated *Chaetopterus* oocytes undergo spontaneous GVBD when placed in NSW. *Spisula* extract at concentrations of 1.0, 2.0, and 3.0 mg/ml inhibited the maturation process by 8, 55, and 69%, respectively. dbcGMP at concentrations of 0.5, 1.0, and 2.0 mM inhibited maturation by 46, 84, and 96%, respectively. dbcAMP was less effective. At concentrations of 1.0 and 2.0 mM, inhibition was 14 and 46%, respectively. Other nucleotides and related compounds including forskolin and IBMX were ineffective or showed less than 10% inhibition.

Spontaneous maturation of mouse oocytes was blocked by HPLC purified *Spisula* factor at a concentration of 5 μg/ml plus 50 μM dbcAMP. The meiotic arresting nucleotide is biologically related to cyclic adenosine 3′,5′-pyrophosphate (cAPP). Moreover, cAPP at a concentration of 10 μg/ml and in combination with 50 μM dbcAMP blocked GVBD. cAPP is a potent inhibitor of cAMP phosphodiesterase and protein kinase (Wood and Lynn 1981, *Differentiation* **18**:51).

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New aspects of the development of the polyclad, Hoploplana inquilina. Louisa M. Rooney and Barbara C. Boyer (Union College).

A method of artificial fertilization was developed for the hermaphroditic polyclad *Hoploplana inguilina* which results in normal development of eggs lacking the impermeable egg shell membrane. This technique greatly facilitiates both chemical and mechanical manipulation of the eggs and embryos. Gametes are released by piercing the uterus (or ovaries), and spermaducal vesicles of a single animal. Eggs and sperm may also be obtained from two different animals. *In vitro* fertilized eggs are raised in penicillin-streptomycin millipore-filtered sea water and form normal larvae in five days.

Hoploplana larvae were cultured through the planktonic stage. The hatchling has six lobes, in contrast to the four-lobed Götte's or eight-lobed Muller's larva. After an average of 15 days in culture no new lobes developed and the original ones regressed as the larvae settled, indicating that this is not a typical Muller's larva. In general no further development occurred except that one metamorphosed worm was first observed 16 days after hatching; it measured 0.6 mm by 0.5 mm and had no eyes or tentacles.

Artificially fertilized eggs were treated with Cytochalasin-B in concentrations of 20, 40, and 80 μg/ml to test its effect on meiotic blebbing and cleavage. The 20 μg/ml dilution had no effect and the 40 μg/ml reduced the rate of blebbing and cleavage and increased abnormalities. The 80 μg/ml dilution completely inhibited both processes. Scanning electron microscopy of untreated eggs revealed microvilli on unblebbed egg surfaces but no microvilli on blebbed surfaces of the same egg. Microvilli are also associated with the first cleavage furrow.

This work was supported by a Research Corporation Grant to B. Boyer.

Modulation of oocyte maturation by membrane components in Spisula. E. SATO (Population Council), S. J. SEGAL, AND S. S. KOIDE.

Maturation of *Spisula* oocytes can be induced with sperm, KCl, or serotonin. We now observed that oocytes treated with trypsin undergo germinal vesicle breakdown (GVBD). Oocytes incubated in 0.05 and 0.1% trypsin solution (type II, bovine pancreas) for 0.5 to 1 h, subsequently washed, and incubated in control medium for 1 to 2 h underwent maturation. Treatment with glycosidase, DNase, and RNase did not induce GVBD. The optimal condition for the induction of maturation with trypsin was to incubate oocytes in 0.1% enzyme solution for 1 h and to transfer the oocytes to a medium composed of 0.4 *M* Tris HCl, pH 7.4, 20 m*M* $CaCl_2$. On the other hand, the frequency of GVBD with trypsin-treated oocytes induced with sperm was less than that with control oocytes, while the response induced with KCl remained unchanged.

To identify the membrane components modulating maturation arrest, oocytes were incubated in a medium containing 1 *M* urea, 5 m*M* EDTA, 10 m*M* Tris HCl, pH 7.4, for 30 min as described previously (Sato, *et al.* 1983, *Gamete Res.* **8**: 119–127). The membrane protein preparation was concentrated, dialyzed, and added to a suspension of trypsin-treated oocytes in 0.4 *M* Tris HCl, pH 7.4, 20 m*M* $CaCl_2$. GVBD was blocked at final concentrations of 0.4 to 0.8 mg of membrane proteins per ml.

Preparation of membrane components was fractionated by gel filtration through Sephadex G-100 column. The activity resided in the fraction corresponding to an estimated MW of 20 kD. The partially purified product was active at a concentration of 0.1–0.2 mg/ml and was partially deactivated on heating at 100°C for 15 min.

These findings suggest that an oocyte membrane component maintains maturation arrest and that removal of this component by a protease triggers resumption of meiosis.

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Isolation and characterization of Spisula *sperm components modified by ^{14}C-gossypol.* HIROSHI UENO (The Rockefeller University), SHELDON J. SEGAL, AND SAMUEL S. KOIDE.

Gossypol inhibits sperm motility. This anti-fertility action together with its reversibility and minimal side effects shown by Chinese scientists makes this yellow cottonseed compound a potential male contraceptive. Its mechanism of action on sperm has not been clarified. However, irreversible inhibition of isolated sperm mitochondrial enzymes with gossypol has been reported. These enzymes include the sperm-specific lactate dehydrogenase-X and various ATPases. In the present study, we determine gossypol binding sites on *Spisula* sperm.

After incubation (10–30 min) of *Spisula* sperm with ^{14}C-gossypol (Sato *et al.* 1983, *Biol. Bull.* **165**: 516–517), excess of either $NaCNBH_3$ or $NaBH_4$ was added to stabilize the adduct at pH 6.0. The unreacted gossypol and reducing agent were removed. The labeled component was solubilized in various media with and without ultrasonication. The most efficient extraction mixture was $CHCl_3/CH_3OH$ (2:1), followed by 5% SDS-10 m*M* EDTA-10 m*M* mercaptoethanol with sonication, 2 *M* urea-10 m*M* EDTA with sonication, phosphate (pH 6.0) or Tris · HCl (pH 7.4) buffer with sonication, and buffer alone without sonication. The recoveries of radioactivity in the supernatant were 60–65%, 40–50%, ~30%, <10%, and none, respectively. The results suggest that the majority of the label was associated with lipid components ($CHCl_3/CH_3OH$ phase) and a fraction of the counts was associated with mitochondrial enzyme fraction (buffer with sonication). To characterize the radioactive adducts the $CHCl_3/CH_3OH$ extract was incubated with phospholipase C (from *C. perfringens*). Change in mobility after phospholipase C treatment was determined by TLC (silica gel; solvent = chloroform-acetone-methanol-acetic acid-water, 6:8:2:2:1). About 50% of the label migrated

with the solvent front and the other 50% stayed in the vicinity of the origin after 5 h incubation, whereas all of the radiolabeled material of the untreated extract remained at the origin. The present results indicate that phospholipids or lipids that are substrates for phospholipase C are the principal acceptors of gossypol in the $CHCl_3/CH_3OH$ extract. This suggests that lipid components, containing phosphatidyl serine, phosphatidyl ethanolamine or aminoethyl phosphonate, are the likely components interacting with gossypol in *Spisula* sperm. Two types of interaction may occur: one through Schiff base formation and the other through hydrophobic interaction. Gossypol modification of membrane components (particularly lipids) may alter mitochondrial enzymic systems involved in ATP synthesis, resulting in the suppression of sperm motility.

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Does maternal RNA have morphogenetic determinant activity in ascidian embryos? SAUL L. ZACKSON (Princeton University).

The yellow crescent cytoplasm of ascidian embryos is believed to contain morphogenetic determinants for larval tail muscle development (Whittaker 1973, *P.N.A.S.* **70:** 2096–2100). Jeffery (1984, *Biol. Bull.* **166:** 277–298) recently showed that certain species of maternal mRNA are localized in the yellow crescent cytoplasm, suggesting that maternal RNA has morphogenetic determinant activity.

Psoralen and its derivatives are compounds which covalently cross-link nucleic acids when activated by long-wave ultraviolet light. To test whether maternal RNA has determinant activity, yellow crescents in 2-cell embryos of the ascidian *Styela clava* were irradiated with 366 nm ultraviolet light in the presence of 1 μg/ml aminomethyltrioxalen (AMT), a psoralen derivative. Irradiated embryos were cultured to the swimming tadpole larva stage, and then were fixed and stained histochemically for acetylcholinesterase (AChE) activity, a zygotically expressed marker for muscle development (Whittaker 1973, *P.N.A.S.* **70:** 2096–2100). Irradiations of 5 to 20 s inhibited tail development: increasing U.V. doses lead to decreasing percentages of normal tadpoles and increasing percentages of tadpoles which failed to hatch or displayed abnormal tail muscles. Abnormal tail muscle morphology was often accompanied by an apparent decrease in AChE activity. In some cases, tadpoles with abnormal tails, and that developed further, proceeded to metamorphose, suggesting that defects were limited to the tail.

In control experiments, 60 s of U.V. irradiation of entire embryos without AMT or treatment of embryos with 1 μg/ml AMT without U.V. irradiation, caused no apparent increase in abnormalities compared to untreated embryos. Entire embryos irradiated for 5 s in 1 μg/ml AMT all failed to hatch.

These results are consistent with the hypothesis that maternal RNA has determinant activity. However, it cannot be ruled out that abnormalities resulted from cross-linking other nucleic acids such as ribosomal RNA or mitochondrial DNA. Finally, the presence of maternal RNA may be a necessary, but not sufficient condition for driving a yellow crescent-containing blastomere toward muscle differentiation.

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NEUROBIOLOGY

Inactivation of voltage-dependent K^+ currents is related to Ca^{2+}-calmodulin-dependent phosphorylation of specific protein. DANIEL L. ALKON (Laboratory of Biophysics, NINCDS-NIH at MBL), SHIGETAKA NAITO, JOSEPH T. NEARY, JUNE HARRIGAN, MANABU SAKAKIBARA, ANDREW WEINER, AND ELIAHU HELDMAN.

Two microelectrode voltage clamps of the *Hermissenda* Type B photoreceptor isolated from synaptic interactions and impulse activity by axotomy, previously indicated that light and depolarization-induced elevation of Ca_i^{2+} cause transient activation of a Ca^{2+}-dependent K^+ current, I_C, and inactivation (lasting minutes) of both I_C and I_A, an early voltage-dependent K^+ current (Alkon *et al.* 1982, *Biophys. J.* **40:** 245–250; Alkon *et al.* 1984, *Biophys. J.* in press). Patch-clamp of the Type B soma in this study revealed: (1) the absence of light-induced inward current (consistent with its origin across the rhabdomeric membrane); and (2) voltage-dependent outward K^+ channel currents which were unaffected by 4-aminopyridine (3 m*M*) and tetraethylammonium ion (100 m*M*) and whose frequency and summated amplitudes increased during illumination. These and other observations (*e.g.*, that I_{Na} and the light-induced I_C have the same action spectrum, *i.e.*, for rhodopsin) suggest that Ca_i^{2+}, elevated by light-induced intracellular release within the rhabdome, must diffuse to the inner surface of the soma membrane to activate and then inactivate I_C channel currents.

Repeated pairing of light and rotation (*i.e.*, conditioning) via its effect on the visual-statocyst neural system causes prolonged Ca_i^{2+} elevation and I_A and I_C reduction which can, with enough pairings, last for

days. A number of observations suggest a role for Ca^{2+}-calmodulin-dependent phosphorylation in reducing I_A and I_C for days. In the present study, eyes isolated from conditioned (*vs.* random or naive controls) at least one day after training showed a significantly ($P < 0.002$) increased ^{32}P incorporation into a protein band of 56,000 M.W. and pI = 5.4, separated by two-dimensional gel electrophoresis. A 24,000 M.W. phosphoprotein (pI = 5.5) may also remain increased during retention. Co-migration studies indicated that the 56,000 M.W. protein has a similar molecular mass, but a very slightly more basic pI than rat tubulin. In broken cell preparations, Ca^{2+} stimulated phosphorylation of a 56,000 M.W. protein (as well as others), and phosphorylation was blocked by the calmodulin inhibitors calmidazolium (50 μM) and trifluoperazine (100 μM), both of which when externally applied, also blocked Ca^{2+}-inactivation of I_C. This is consistent with an earlier finding that intracellular iontophoresis of an exogenous Ca^{2+}-calmodulin-dependent protein kinase enhanced and prolonged Ca^{2+}-inactivation of I_C as well as I_A. The role of the 56,000 M.W. phosphoprotein, as well as other proteins of interest (*e.g.*, 20–25,000 M.W. range), in the regulation of K^+ channel function remains to be determined.

Catecholamine neurons in normal and deafferented toadfish (Opsanus tau) *olfactory bulb and telencephalon.* H. BAKER AND M. WEISER (Department of Neurology, Cornell University Medical College, New York, New York).

The olfactory bulbs of higher vertebrates contain dopamine neurons demonstrable with antibodies against tyrosine hydroxylase (TH), the first enzyme in the catecholamine biosynthetic pathway. We find that putative dopamine neurons can be localized with TH antibodies in the olfactory bulb of the toadfish (*Opsanus tau*). In contrast to the mammal, the toadfish telencephalon also contains numerous TH stained neurons. These appear to be contiguous with those of the olfactory bulb. The relationship of peripheral afferent input to the dopamine neurons of the olfactory bulb and telencephalon was studied by applying WGA-HRP to the peripheral end organ (the receptor epithelium) or HRP to the central cut end of the olfactory nerve 4–6 mm rostral to the olfactory bulb. Both techniques labeled glomeruli within the olfactory bulb, but not the ipsilateral telencephalon. These studies demonstrate that the dopamine neurons of the telencephalon are not directly related to those of the olfactory bulb and its afferent input. In addition, we investigated in the toadfish the phenomenon observed in the mammalian olfactory bulb of a loss in TH staining, and thus dopamine synthetic capacity, following peripheral deafferentation. Central olfactory nerve lesions were performed and 2–5 weeks postsurgery the toadfish were analyzed for TH immunoreactivity. Staining for TH remained in both the telencephalon and olfactory bulb; however, the deafferented fish olfactory bulb, like the mammalian, appeared reduced in size reflecting the loss of afferent input. We conclude that dopamine neurons in the toadfish olfactory bulb retain their ability to synthesize the enzyme tyrosine hydroxylase following peripheral deafferentation. The lack of change in transmitter enzyme synthesis in the teleost may result, in part, from a species difference in connectivity between receptor afferents and catecholamine neurons. In addition, the larger regenerative capacity of teleost neurons, as compared to mammalian, may depend on continued transmitter synthesis in deafferented cells producing a more stable target for new axonal growth and terminal aborization.

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Vision in Limulus *mating behavior during the day and at night.* ROBERT B. BARLOW, JR., MAUREEN K. POWERS, LEONARD KASS, ROBERT W. FIORDALICE, MARK D. CAMARA, AND HEIDI A. HOWARD (Marine Biological Laboratory).

During mating season, male horseshoe crabs can use vision to find females (Barlow *et al.* 1982, *Nature* **296:** 65–66). Males will approach high-contrast cement castings of the female carapace and other forms. We showed previously that during the day males moving within 1.2 m of a submerged black hemisphere (29 cm diameter) oriented toward the cement form and contacted it (Barlow *et al.* 1983, *Biol. Bull.* **165:** 539). Since mating occurs primarily at night, we report here studies of the visually-guided behavior of males in the vicinity of the black hemisphere both during the day and at night.

We recorded the animals' movements on video tape, using an infrared illumination and TV system. We studied 523 males in seven daytime and six nighttime sessions on Mashnee Dike between 22 May and 14 June, 1984. Their behavior was divided into two categories according to whether the animals made contact with the black hemisphere. For males that made contact, we measured the distance from the hemisphere at which the animals oriented toward it. For males that did not make contact, we measured the distance between the hemisphere and the animal's point of closest approach.

Measurements show that during the day some animals orient toward the hemisphere as far away as 1.2 m, which agrees with our previous measurements cited above. At night, animals must approach within 1.0 m before they can see the hemisphere. At these distances, the base of the hemisphere subtended visual angles of 14 and 17 degrees respectively for day and night orientation. Measurements of the eye's optical

resolution (Barlow, unpubl. obs.) indicate that objects of this size can be seen by only four visual receptors during day and night. For all males that contacted the hemisphere, the average distances at which orientation occurred were 0.97 0.04 m and 0.53 0.04 m respectively for day and night.

We conclude that the coarsely-faceted compound lateral eyes of *Limulus* allow males to find females both day and night. The circadian changes in the lateral eye's sensitivity (Barlow 1983, *J. Neurosci.* **3**: 856–870) may play an important role in the animal's visually-guided behavior.

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Effects of behavioral activation of the efferent vestibular system on the response dynamics of primary afferents of the horizontal semicircular canal in the toadfish, Opsanus tau. R. BOYLE AND S. M. HIGHSTEIN (Marine Biological Laboratory, and Department of Otolaryngology, Washington University School of Medicine, 4911 Barnes Hospital Plaza, St. Louis, Missouri 63110).

Response dynamics of primary afferents of the horizontal semicircular canal (HSC) to angular acceleration and efferent activation were studied with glass microelectrodes in unanesthesized toadfish. Adult toadfish (*ca.* 500 gm) were perfused through the mouth with running sea water. Dorsal craniotomy (less than 6 mm diameter) exposed the HSC nerve. The magnetic search coil technique measured eye position which served as a behavioral marker. The tank, manipulators, and preamplifiers were mounted on a rate table for sinusoidal rotation about the vertical axis with the center of rotation between the two HSCs. Background discharge in absence of rotation of over 400 fibers ranged from zero to low rates (less than 10 ips) with irregular interspike intervals, to higher rates (greater than 80 ips) with regular intervals, as reported for other vertebrate labyrinths. Sinusoidal rotation (0.01–15.0 Hz) at various amplitudes (±0.5°–±45°; 2–30°/s; 0.2–600°/s^2) determined the response dynamics, *i.e.*, sensitivity and phase re: acceleration and velocity, including corner frequencies, of studied afferents. Impulse activity in identified efferent vestibular fibers can be enhanced by a light touch to the snout that also produces behavioral activation *e.g.*, eye retraction. This facilitates primary vestibular afferents presumably via excitatory axo-axonic synapses (Highstein and Baker 1983, *Biol. Bull.* **165**: 527; Sans and Highstein, *Brain Res.* in press). In this study efferent activation was evoked by a light touch combined with angular acceleration to produce consistent facilitation in afferent discharge. Afferent fibers having low resting rates were more strongly affected by the efferent action than regularly discharging afferents. Efferent activation resulted in an advance and/or prolongation of the excitatory phase of the response to rotation, adding between 2–25 ips to this half-cycle. It also evoked increased discharge in the inhibitory off-direction. Afferent impulses were often seen during behavioral activation within the stimulus period wherein angular acceleration alone would normally silence the fiber. However, the level of behavioral arousal of the fish was a critical factor in evoking the efferent action, and not all fish responded to the experimental paradigm. In conclusion, the background discharge and response dynamics of HSC afferents resemble those observed in other vertebrates. Further, the facilitatory action of the efferent vestibular system on primary afferents evident in the toadfish approximates that seen using electrical stimulation in the squirrel monkey. This efferent action can alter the response characteristics of afferents to angular accelerations.

Control of light emission from Obelia *photocytes.* PAUL BREHM AND KATHLEEN DUNLAP (Tufts School of Medicine).

Cellular control of *in situ* calcium-activated photoprotein was examined in light emitting cells (photocytes) from *Obelia geniculata.* Tissue from *Obelia* was dissociated by trituration and maintained in MBL artificial sea water. Photocytes containing the calcium-activated photoprotein were identified by the brilliant fluorescence produced by the photoprotein/green fluorescent chromophore complex. Photocytes in intact tissue were irregular in shape. Following dissociation they became generally spherical with diameters ranging between 10 and 30 microns. Membrane currents were monitored in both photocytes and nonphotocytes by the whole cell voltage-clamp technique. In photocytes, step depolarizations to potentials above 0 mV (from holding potentials below −30 mV) evoked outward currents which reached their peak within a few ms and inactivated fully during 200 ms pulses. No voltage dependent inward current was observed in any of 25 photocytes tested. Additionally, under whole cell recording, no luminescence was elicited from isolated photocytes. In experiments where small clumps of cells containing a single photocyte were tested different results were obtained. Depolarization of a nonphotocyte by either extracellular stimulation or by whole cell voltage clamp elicited light flashes in the neighboring photocyte. Additionally, at potentials which caused light emission from the photocyte, voltage dependent inward currents were observed in the nonphotocyte. These results indicate that nonphotocytes are required for production of light from photocytes. Additional support for this idea came from the observation that application of isotonic KCl to 14 isolated photocytes did not stimulate light emission, whereas application to 31 clumps of cells containing a single

photocyte evoked light production in 24 trials. Our data suggest that nonphotocytes serve as an inward current generator, propagating the action potential throughout the electrically coupled epithelium. The calcium necessary for activation of the photoprotein may result from a synaptic interaction between photocytes and nonphotocytes rather than from direct depolarization of the photocyte by adjacent tissue.

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Enkephalin, opiate of the sperm cell: the Neapolitan connection. LUCIO CARIELLO (Stazione Zoologica, Naples, Italy) AND LEONARD NELSON.

Sea urchin sperm exposed to morphine appear, in the microscope, to swim more vigorously and for a longer time than do untreated controls. Rates of forward progress quantitatively determined on centrifugally oriented cells were both dose- and time-dependent. *Arbacia* spermatozoa were incubated for 0, 5, 19, and 15 minute periods in artificial sea water solutions of methionine enkephalin acetate and in naloxone HCl. The sperm showed biphasic increases in speed up to 200% and 175% of the control rate at 0.1 m*M* and 0.5 m*M*, respectively, after 15 min treatments. When incubated simultaneously in varying concentrations of the opiate and a fixed concentration of the antagonist at a subliminal level (16 μM), the stimulatory response was abolished over the entire concentration range (1 μM–1 m*M*). The nature of these responses illustrates the specificity of the opiate peptide effect on sea urchin sperm. During the course of purification of acetylcholinesterase extracted from sperm flagella, radioimmunoassay performed on rat brain synaptosomes showed the presence of opioids in the sperm flagellar membranes. The integration of the opiate peptide system into the activity of the cholinergic complex that regulates flagellar motion is strikingly emphasized by the recent demonstration that beta-endorphin, and to a lesser extent, metenkephalin, specifically inhibit motor endplate acetylcholinesterase (Haynes and Smith 1982, *Neuroscience* **7**: 1007–1013). Regulation of sperm cell swimming performance by an intrinsic cholinergic system thus appears to be susceptible to the influence of an intracellular, naturally occurring neuropeptide.

This work was supported by NSF research grants and by CNR.

Transport of Na^+ inside the giant axon of squid. D. C. CHANG (Baylor College of Medicine, Houston, Texas 77030).

We injected ^{22}Na into the giant axon of squid to study how Na ions are transported within the nerve cell. The purpose of this study is to test an hypothesis that, in addition to the active transport across the cell membrane, the nerve cell may utilize axonal transport to maintain a low concentration of Na^+ inside the axon. We know that not all intracellular ions are free. Studies using ion-selective micro-electrodes have indicated that approximately ⅓ of the intracellular Na^+ are osmotically inactive. Some of the axoplasmic Na^+ could be sequestered into vesicles which are moved by axonal transport. To test this scheme, ^{22}Na ions were injected into the axon and allowed to diffuse for 2 to 6 hours, while the axon was kept under oil or natural sea water. Then, the distribution of ^{22}Na along the axon was determined by measuring the profile of radioactivity.

The results suggest that the axoplasmic Na^+ are not irreversibly sequestered into the vesicles involved in axonal transport. The distribution of ^{22}Na follows a smooth bell-shaped curve peaked at the location at which the ^{22}Na was injected. If a significant number of injected ^{22}Na ions were irreversibly sequestered into vesicles and then moved by the fast axonal transport, we would have observed a shift of the ^{22}Na profile toward the direction in which the vesicles move. Such effect was not observed.

The results of our measurements indicate that the transport of Na^+ within the axon is dominated by diffusion. From our data, the diffusion coefficient of Na^+ in the axoplasm is estimated to be $(8.2 \pm 3.5) \times 10^{-6}$ cm^2/s at 4°C, and $(12.7 \pm 3.3) \times 10^{-6}$ cm^2/s at room temperature.

This work is partially supported by an ONR contract. The assistance of Jessica Liu is also acknowledged.

Detection of an inositol 1,4,5-trisphosphate-induced rise in intracellular free calcium with aequorin in Limulus *ventral photoreceptors.* D. WESLEY CORSON, ALAN FEIN, AND RICHARD PAYNE (Marine Biological Laboratory).

Inositol 1,4,5-trisphosphate ($InsP_3$) is thought to be a second messenger which releases calcium from endoplasmic reticulum. Injection of $InsP_3$ into ventral photoreceptors results in both excitation and adaptation (Fein *et al.* 1984, *Nature* in press; Brown *et al.* 1984, *Nature* in press). Light adaptation in *Limulus* photoreceptors appears to result from a rise in intracellular free calcium (Ca_i). We attempted to detect an $InsP_3$-induced rise in Ca_i by using the calcium indicator protein aequorin.

Aequorin and $InsP_3$ were kindly provided by Dr. O. Shimomura and Drs. R. F. Irvine and M. J. Berridge, respectively. Ten to forty picoliters of solution containing 7.2 mg of aequorin/ml, 50 m*M* K aspartate, 5 m*M* HEPES (pH 7.0) and 50 μM EGTA were injected into 9 photoreceptors. Subsequent injections of 1 m*M* $InsP_3$ (with 100 m*M* K aspartate and 10 m*M* HEPES, pH 7.0) produced a depolarization

of the membrane and aequorin luminescence detected with a photomultiplier. Not every $InsP_3$-induced depolarization was associated with a rise in Ca_i; either because a rise in intracellular calcium did not always occur; or, more likely, because our detection system was not sufficiently sensitive. The peak of the aequorin response (time to peak, tp = 578 ± 112 ms) usually preceeded the peak of the $InsP_3$-induced depolarization (tp = 620 ± 112 ms) but lagged behind in 8 measurements out of 26. In 10 measurements in 7 cells, the peak of light-evoked luminescence (tp = 280 ± 28 ms) never preceeded the peak of the light-induced depolarization (tp = 208 ± 40 ms). These measurements demonstrate directly, for the first time, that injection of $InsP_3$ into living cells results in a rise in Ca_i. The rise in calcium appears to be fast enough to participate in adaptation and perhaps in excitation as well.

Sex discrimination in lobsters: urine cues. DIANE COWAN AND JELLE ATEMA (Boston University Marine Program, Marine Biological Laboratory).

Behavioral studies indicated that mature female lobsters, *Homarus americanus*, may use chemical cues to establish and maintain a five to ten day pair bond in the male shelter. In four 2000 l aquaria the responses of one female and two male lobsters to urine and body odor were observed in a seminatural environment where lobsters occupied observation shelters. Stimuli were injected once or twice per day into a continuously flowing delivery tube attached to the shelters. Habituation to stimulus introductions was avoided by the natural interactions between the three lobsters in each tank. Durations of seven different behaviors were recorded in blind tests. Food satiated male and female lobsters were presented with either male or female urine or body odor. Controls included home tank water stimuli and pre-trial observations. Results were compared (*t*-test) to controls and to each other for sex and odor differences.

The most commonly observed behavior in response to all odors was checking the entrance with closed claws while pleopod fanning; it represents accurately the response to an approaching lobster in nature. The behavior most commonly scored in odor communication studies with isolated animals, locating the stimulus source, was not significant here. Female lobster urine introduced into male shelters elicited the greatest response. In general, lobsters responded more to urine than to body odor. Males were more responsive than females to all of the odors tested. Males responded to the odor of freshly molted females by standing high on their walking legs; no other odor elicited this response in males or females. This male behavior is characteristic during cohabitation.

The bioassay developed here is capable of differentiating between responses in a biologically relevant context. Hence, it may be used to identify compounds of social importance to lobsters.

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Mauthner cells, Muller cells, and the lamprey startle response. SCOTT CURRIE (University of California, Davis).

Preliminary work with intact larval lampreys (*Petromyzon marinus*) revealed a rapid, vibration-evoked startle response involving both sides of the body (Currie 1984, *Neurosci. Abstr.* **10**). A preparation has been developed to allow a cellular analysis of this behavior. The brain and spinal cord are dorsally exposed while the lateral body musculature is left intact. A fine steel probe is used to deliver 3 cycles of 300 Hz. vibration to an exposed auditory capsule. Such a stimulus produces a volley of large, descending units in the spinal cord followed by a robust, bilateral electromygram (EMG) response.

In a series of curarized preparations, fine-tipped suction electrodes were used to record the extracellular spikes of identified reticulo-spinal neurons, visable beneath the surface of the fourth ventricle. Vibration-evoked units were seen over both Mauthner and both B_1 Muller cells. Subsequent intracellular recording showed these, as well as both B_2 cells, to be strongly depolarized by vibration. Several other Muller neurons, which had been silent extracellularly, were seen to be inhibited (ipsilat. I_1, both B_3's, both B_4's). Labyrinthectomizing the vibrated auditory capsule prevented these effects.

Slight modifications of this preparation made it possible to record intracellularly during the startle response. In one experiment, both Mauthner axons were impaled near the brain. Vibration elicited synchronous spikes in both axons followed by the bilateral muscle response. Single action potentials in either axon, elicited by intracellular current injection, produced smaller bilateral responses. Simultaneous stimulation of both Mauthner axons exactly reproduced the vibration-evoked behavior.

Supported by a Grass Fellowship. Special thanks to Dr. T. H. Bullock, in whose laboratory most of the early work was done.

Voltage clamp studies of the terminal region of barnacle photoreceptors. RALPH E. DAVIS, JOHN W. MOORE, AND ANN E. STUART (University of North Carolina, Chapel Hill).

Preliminary experiments measured currents through calcium and calcium-activated potassium channels (CaGk) in the synaptic terminals of photoreceptors in *Balanus nubilus*. The terminal region and a short

segment of the receptor axons was isolated from the main length of the ocellar nerve and somata by passing the nerve through a barrier and sealing it with Vaseline. A microelectrode sensed the photoreceptor membrane potential in the axon near (.1 to 1 mm) the release sites. A feedback circuit controlled the measured potential by injecting current which entered the axons in the ocellar pool.

When the terminal region was perfused with normal saline (containing 20 m*M* calcium), depolarizing voltage steps produced large currents (presumably potassium current) which showed small dips. When this large current was blocked by the addition of 20 m*M* TEA, clear inward currents followed by outward currents (developing over tenths of seconds) were observed. Reduction of the calcium concentration to 8 m*M* resulted in improved voltage control.

The inward current was identified as flowing through calcium channels and the outward current as flowing through CaGk channels by substitution of barium and cobalt for calcium while maintaining the TEA at 20 m*M*. In barium saline the inward currents were prolonged and no outward current developed. In cobalt both inward and outward currents were blocked.

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Conditioning causes intrinsic membrane changes of rabbit hippocampal neurons in vitro. JOHN F. DISTERHOFT (Marine Biological Laboratory), DOUGLAS A. COULTER, AND DANIEL L. ALKON.

The hippocampus is importantly involved in learning in the human and other mammalian species. We studied hippocampal pyramidal neurons from brain slices of conditioned, pseudoconditioned, and naive rabbits. Three 80-trial sessions of nictitating membrane conditioning or pseudoconditioning were carried out. Twenty-four hours after training, slices of the left hippocampus were prepared and maintained using standard procedures.

Pyramidal neurons from the CA1 subfield were penetrated with 80–100 MΩ electrodes filled with 3 *M* KCl. These neurons were ortho- and antidromically identifiable by stimulation of the Shaffer collaterals and the alveus, respectively. All neurons included in this report showed stable penetrations with at least 60 mV spikes. Thus far, 13 conditioned, 10 pseudoconditioned, and 15 naive neurons have been studied.

No difference among groups was seen in membrane resting potential, spike height, membrane input resistance, or EPSP size to orthodromic stimulation. Differences were present in two responses. (1) The amount of afterhyperpolarization (AHP) seen after a depolarizing current induced spike decreased significantly in conditioned ($X = -1.13$ mV) as compared to pseudoconditioned ($X = -2.04$ mV; $P < .025$) and naive ($X = -2.12$ mV; $P < .01$) cells. (2) The amount of "sag" observed when 100 ms, 1 na hyperpolarizing current pulses were injected into the cells was reduced in the conditioned ($X = 1.77$ mV) as compared to the pseudoconditioned ($X = 2.70$ mV; n.s.) and naive ($X = 5.67$ mV; $P < .001$) cells.

The AHP in hippocampal neurons has been demonstrated to be caused by a Ca^{++}-mediated K^+ current. Its reduction after conditioning would make the pyramidal cells more excitable. A similar current, I_c, is reduced in *Hermissenda* after conditioning. Our data demonstrate the feasibility of analyzing the cellular mechanisms of associative learning in mammalian brain with the *in vitro* brain slice technique. They also suggest that similar mechanisms may subserve conditioning in the invertebrate and mammalian nervous systems.

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Information flow in phototransduction; from rhodopsin to a GTP-binding regulatory protein to phospholipase-C. ALAN FEIN, D. WESLEY CORSON, AND RICHARD PAYNE (Marine Biological Laboratory).

It has been proposed that phototransduction in *Limulus* ventral photoreceptors involves the activation of a GTP-binding regulatory protein (N) by photoactivated rhodopsin (Corson and Fein 1983, *J. Gen. Physiol.* **82:** 639–657). Recently it was discovered that injection of inositol 1,4,5-trisphosphate ($InsP_3$) both excites and adapts ventral photoreceptors (Fein *et al.* 1984, *Nature* in press; Brown *et al.* 1984, *Nature* in press), which suggests that liberation of $InsP_3$ by phospholipase-C may be involved in phototransduction. These findings lead to the hypothesis that rhodopsin activates N which in turn activates a phospholipase-C to release $InsP_3$ and excite the photoreceptor. If this hypothesis is correct then blockade of the transduction pathway at N should not affect excitation by $InsP_3$. We tested this possibility by introducing GDP-β-S [Guanosine 5′-O-(2-thiodiphosphate)] into the cell to inhibit N (Eckstein *et al.* 1979, *J. Biol. Chem.* **254:** 9829–9834). Ventral photoreceptors were impaled with two electrodes; one containing 20 m*M* GDP-β-S in a stock solution of 100 m*M* K Asp, 10 m*M* Hepes, pH 7.0, the other containing 100 μM $InsP_3$ in the same stock solution. Either solution could be injected into the cell by pressure pulses. The cell was excited

first by a flash of light and then by an injection of $InsP_3$. The cell was then injected with GDP-β-S, after which the amplitude of the response to light was attenuated more than ten-fold, while the amplitude of the $InsP_3$ response was essentially unaffected. These findings suggest that if the response to light is mediated by release of $InsP_3$, then the site of action of GDP-β-S precedes this release.

Synaptic potentials in antidromically identified oculomotoneurons in the winter flounder, Pseudopleuronectes americanus. W. GRAF AND R. BAKER (The Rockefeller University and New York University Medical Center, New York, New York).

Vestibulo-ocular reflex pathways in mammals are generally organized in a reciprocally innervated fashion. Oculomotoneurons to vertical eye muscles largely receive excitatory synaptic input from the contralateral, and inhibitory input from the ipsilateral semicircular canals. Although this innervation scheme has been established in mammals and was also reported in amphibians, available studies in fish failed to demonstrate inhibitory postsynaptic potentials (IPSPs). By contrast, both chemical and electrical excitatory postsynaptic potentials (EPSPs) have been observed. Nonetheless, our prior morphological studies of individual HRP identified second order vestibular neurons in the winter flounder which strongly inferred the existence of excitatory as well as inhibitory neurons like those observed in mammals. We, therefore, recorded the postsynaptic potentials in antidromically identified oculomotoneurons following electrical stimulation of single vestibular nerve branches. In well identified intrasomatic penetrations, EPSPs were always elicited from stimulation of the appropriate contralateral labyrinthine nerve, yet ipsilateral vestibular stimulation did not yield any significant membrane hyperpolarization at normal resting potentials. However, in some cells, but not all, IPSPs could be demonstrated when the soma was presumably slightly injured or depolarized by injection of depolarizing current in the absence of action potentials. In these cases, the IPSP could also be reversed by injection of hyperpolarizing current. These data suggest that the equilibrium potential for the IPSP in fish oculomotor neurons is close to the resting membrane potential. On the other hand, intrasomatic recording may not reflect well the extent of inhibitory conductance because HRP reconstructed motoneurons exhibit long radiating dendritic fields that argue against a uniform change in potential throughout all compartments. These findings suggest that further study of intradendritic recordings including conductance measurements will be necessary to specify the exact interaction between the excitatory and inhibitory synaptic potentials.

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Mating behavior of Limulus: *relation to lunar phase, tide height, and sunlight.* HEIDI A. HOWARD, ROBERT W. FIORDALICE, MARK D. CAMARA, LEONARD KASS, MAUREEN K. POWERS, AND ROBERT B. BARLOW, JR. (Marine Biological Laboratory).

Horseshoe crabs (*Limulus polyphemus*) move in from deep water in the spring and build nests near the water's edge on protected beaches along the eastern coast of North America. To study the dynamics of this mating activity we mapped out three transects (10 m $\times$ 10 m) on the south side of Mashnee Dike, Cape Cod, Massachusetts. The transects were separated by 10 m and each extended 10 m into the water regardless of tide height. We counted the number of animals within these transects as a function of the time of day from late spring until mid-summer, 1984.

Mating activity was observed from 14 May until 3 July 1984. The greatest number of animals appeared within the transects during the time of the full and new moons, but significant numbers also appeared at other lunar phases. Peaks of activity were observed near the times of the two daily high tides. The maximum number of animals generally occurred about one hour after high tide. Virtually no animals were observed in the transect during low tides.

The number of animals in the two daily peaks in mating activity are not equal, and the difference appears to be correlated with the semidiurnal inequality of the high tides. During May and June at Mashnee Dike, the higher of the two tides occurs in the afternoon and at night (1445–0215 hours). We found that the greater number of animals populate the higher of the two high tides except when it occurs in the late afternoon (1445–1715 hours). On these occasions, the animals remain with the lower high tide (0245–0500 hours) until it occurs after dawn (later than 0500 hours). At that point, the animals switch to the late afternoon high tide. In summary, *Limuli* preferentially populate the higher of the two daily high tides unless the lower high tide occurs before dawn. We conclude that lunar phase, tide height, and sunlight all appear to influence the mating behavior of *Limulus.*

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Evaluation of a new tetrodotoxin preparation. S. L. HU AND C. Y. KAO (Department of Pharmacology, State University of New York Downstate Medical Center, Brooklyn, New York 11203).

The Sankyo Company of Japan has been the only commercial source of tetrodotoxin (TTX). Because of the importance of tetrodotoxin as a neurobiological and physiological tool, an alternative source of supply may be beneficial in terms of cost, quality, and availability.

The Fisheries Research Institute of Hebei Province, Peoples Republic of China, has recently isolated and purified tetrodotoxin on a commercial scale. We have tested the neurobiological actions of this product on the voltage-clamped, internally perfused squid giant axon, and on the single frog (*Rana pipiens*) skeletal muscle fiber in a vaseline-gap voltage-clamp method.

On both preparations, the Hebei TTX is selective in blocking the inward and outward sodium current, with no apparent alteration of the kinetics of activation and inactivation, and of the voltage-dependence of the sodium conductance. On the frog muscle, the ED_{50} determined on the basis of residual sodium current is 3.68 n*M* for the Hebei TTX and 4.10 n*M* for the Sankyo TTX. With the Hebei TTX, 75–92% of the original I_{Na} can be recovered in about 10 minutes of wash, as opposed to 70–80% with the Sankyo TTX. Therefore, the Hebei TTX can be an acceptable alternative to the currently used Sankyo preparation.

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Voltage-dependent chloride conductance of toad skin: localization by vibrating probe. URI KATZ (Israel Institute of Technology) AND CARL SCHEFFEY.

The ionic transport pathways of amphibian skin help regulate serum ionic content. The system is an excellent model for tight epithelia in general. The skin of the toad *Bufo viridis* has voltage-dependent chloride conductance which activates over tens of seconds upon hyperpolarization (Katz and Larsen 1984, *J. Exp. Biol.* **109:** 353). We used the vibrating probe in combination with Ussing chamber (Jaffe and Nuccitelli 1974, *J. Cell Biol.* **63:** 614; Scheffey *et al.* 1983, *J. Membr. Biol.* **75:** 193) to localize this chloride pathway on the surface of the epithelium. Skin of toads adapted to salt water (Katz and Larsen 1984, *J. Exp. Biol.* **109:** 353) were bathed in toad ringer on both sides and hyperpolarized (outside negative) and scanned with the vibrating probe. Peaks of current density were observed only over a portion of the mitochondrion-rich cells. No other peaks of current density were observed and many of the mitochondrion-rich cells had no measurable current associated with them. The current at mitochondrion-rich cells was abolished by replacement of chloride ions with nitrate ions in the outside bath. After a hyperpolarizing step from short circuit, it activates with the same kinetics as the known voltage-dependent chloride conductance. Our preliminary conclusion is that the voltage-dependent conductance of salt water adapted toads is localized to mitochondrion-rich cells.

This work was done at the Vibrating Probe Facility at MBL and was supported by NIH grant RR01395-03 and by the Fund for Basic Research of the Israel Academy of Sciences and Humanities.

Optical measurements of activation and inactivation of sodium channels. DAVID LANDOWNE (University of Miami).

Squid axons were internally perfused and voltage-clamped between crossed polars. Light passing through the polars and axon was measured with a photodetector. During an 8 ms pulse from −80 to 0 mV there is a rapid (0.3 ms) decrease in light and then a slow decrease for the rest of the pulse. At the end of the pulse there is a rapid increase about ⅔ the amplitude of the initial decrease and then a slow return to the baseline. During this slow recovery the amplitude of the rapid birefringence response to brief test pulses also slowly recovers to the initial level. The behavior of the rapid phase directly parallels the immobilization of polarization ('gating') currents and the inactivation of ionic sodium currents. The slow off phase has also been recognized in the polarization currents.

The rapid activation phase of the birefringence response precedes the increase in sodium permeability. The time course of the fast and slow birefringence changes are voltage dependent, as is the delay between activation and opening. This suggests a model with three states of the sodium channel molecule: resting, activated, and inactivated. The aqueous ionic channel can form or open from the activated state in a voltage-dependent step and closes either to the activated or the inactivated state with a rate that is independent of voltage. Computations based on this kinetic scheme simulate the behavior of the ionic and polarization currents of the sodium channel.

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Regional properties of calcium entry in a barnacle neuron. LISA A. LEWENSTEIN AND WILLIAM N. ROSS (New York Medical College, Valhalla, New York).

With an array of photodiodes we examined calcium influx from different positions on identified neurons of the supraesophageal ganglion of *Balanus nubilus* injected with the calcium indicator dye Arsenazo III. The "cross-commissural" cell in this ganglion was capable of supporting a propagating calcium action potential in 3×10^{-7} *M* TTX saline or in sodium-free saline. Propagation persisted when the external calcium concentration was reduced from normal (20 m*M*) to 5 m*M* or less. In normal saline and in TTX saline, absorbance changes of the dye at 640 nm, corresponding to calcium entry, were detected along this cell's axon extending out the nerve root (a distance of 1200 μm from its soma). The signal remained constant in magnitude over a 600 μm length of axon of uniform diameter and increased over areas of neuropile. These larger signals had a faster time course for decay to baseline than the signals over the axon. This may be due to the greater surface area-to-volume ratio in smaller processes where calcium would remain closer to the membrane and nearer to the membrane pumps. Other explanations include more membrane pumps or higher calcium buffering in the neuropile region.

In one experiment both the cell body and axon (600 μm from the soma) of the cell were impaled with microelectrodes. In 10 m*M* TEA the amplitude and duration of the axonal action potential increased. Since TEA blocks voltage-dependent potassium channels this experiment shows that these channels exist in the membrane. Therefore, the propagating calcium action potential is most likely due to a high density of calcium channels instead of a low density of potassium channels.

Calcium channels exist in the somata and presynaptic terminals of many neurons, but are generally thought to be of low density in the axon. The "cross-commissural" cell is a counterexample.

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Transmitter release by local increase in $[Ca^{++}]_o$ *and by prolonged presynaptic voltage steps in the squid giant synapse.* R. LLINÁS, M. SUGIMORI, AND C. S. LEONARD (New York University School of Medicine).

Transmitter release was determined in the giant synapse by simultaneous pre- and postsynaptic voltage clamping after g_{Na} and g_K blockage by TTX and by Cs, TEA, and 3-AP, respectively. The relation between Ca^{++} current (I_{Ca}) and the amplitude and rate of rise of postsynaptic voltage or current (PSP or PSI) was determined. $[Ca^{++}]_o$ was then reduced to 0.2 m*M* in the bath and was locally increased at the most distal portion of the presynaptic terminal digit by pressure injection pulses from a microelectrode located 50–100 μm from the terminal and filled with 100 m*M* $CaCl_2$ or 500 m*M* $Ca(acetate)_2$. A discrete injection of Ca^{++} produced, after a few seconds, a return of synaptic release. I_{Ca} under these conditions was similar to that observed prior to the reduction of $[Ca^{++}]_o$. Synaptic release gradually returned to baseline in 6–12 min after the injection. The relation between I_{Ca} and PSP or PSI had slopes ranging from 1.1 to 2.2. In one case a steeper slope was found. The slope of this relation was similar during both the rising and falling of I_{Ca} as $[Ca^{++}]_o$ changed after injection.

In a second set of experiments the rate of fall of PSPs was determined using long (6, 60, and 600 ms) voltage clamp pulses (10–20 mV). The time course and the time constant of the PSP decay (3.3 ms) did not change even after 600 ms of inward Ca^{++} flow.

The following conclusions are derived from these experiments: (1) The slope of I_{CA} *versus* PSP varies between synapses indicating that such slope does not represent a simple stoichiometric relation between Ca^{++} and vesicle release but rather must involve more than one functional compartment. (2) Since no increase in the duration of the "off" EPSP was detectable even after 600 ms of continuous release, no evidence of "residual calcium" capable of releasing transmitter was found. Together, these results suggest that synaptic transmission occurs during I_{Ca} (when $[Ca^{++}]_i$ is large) and that facilitation must operate on a mechanism different from that of release, *e.g.*, by increasing transmitter availability.

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Optical recording of neuronal activity of the buccal ganglion of Navanax *during feeding.* J. A. LONDON (Yale University School of Medicine), D. ZECEVIC AND L. B. COHEN.

We are studying the neuronal basis of simple forms of learning. Using optical methods for simultaneous monitoring of activity from many neurons, we hope to be able to identify all the altered interactions.

A "whole" animal preparation of the marine slug, *Navanax inermis,* was used to allow simultaneous recording of behavior and neuronal activity. An incision was made in the dorsal surface of the animal exposing the buccal ganglion and pharynx. The buccal ganglion was pinned to a 1 mm light guide that passed through incisions in the ventral body wall and pharynx. The image of the ganglion fell on a 124

element photo-diode array. The ganglion was stained with a voltage sensitive dye, Rh155b. To reduce movement artifact the ganglion was covered with a 3% solution of agar. The feeding behavior was recorded on video tape.

During spontaneous expansions activity was detected in as few as 5 neurons and as many as 19 neurons of a hemi-ganglion. During one food-elicited expansion where the food was injested, we detected activity in 27 neurons.

At the end of the experiment we severed the nerves of the buccal ganglion and electrically stimulated 6 of these to determine the fraction of cells whose activity could have been detected. In our best experiment, we detected activity in 112 neurons. In preliminary counts of the total number of neurons in 2 hemi-ganglia we found an average of 155 neurons. Thus, our optical recording detected activity in approximately 70% of the neurons. We think we have developed a whole animal preparation in which we are recording from a substantial fraction of the number of neurons generating a part of the feeding behavior.

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Possible roles of synapsin I and Ca^{++}/calmodulin-dependent protein kinase II in synaptic transmission as studied in the squid giant synapse. T. L. McGUINNESS, C. S. LEONARD, M. SUGIMORI, R. LLINÁS, AND P. GREENGARD (Rockefeller University and New York University School of Medicine).

Synapsin I is a neuron-specific phosphoprotein localized in presynaptic terminals in association with synaptic vesicles within the mammalian nervous system and is a major substrate for cAMP-dependent protein kinase and two Ca^{++}/calmodulin-dependent protein kinases. The state of phosphorylation of synapsin I can be altered by a variety of physiological and pharmacological stimuli known to affect synaptic function. Phosphorylation of synapsin I by Ca^{++}/calmodulin-dependent protein kinase II (CaM kinase II) has been shown to decrease the affinity of the molecule for synaptic vesicles.

In the present study, the possible roles of synapsin I and CaM kinase II in synaptic transmission were examined in the squid giant synapse. Immunoblot analysis demonstrated that immunoreactive species of both synapsis I and CaM kinase II are present in the squid nervous system. Moreover, CaM kinase II activity was demonstrated in crude homogenates of squid stellate ganglion tissue.

To test directly what effect synapsin I and CaM kinase II might have on synaptic transmission at the squid giant synapse, the purified proteins were pressure injected into the presynaptic terminal digit of the giant synapse under voltage clamp conditions. Prior to injection, the Na^+ and K^+ conductances were blocked with TTX and 3-AP. The presynaptic terminal was voltage clamped and depolarization steps of different amplitudes were delivered before and at various times after injection of either synapsin I or CaM kinase II. Injection of synapsin I resulted in a reproducible decrease in the postsynaptic potential without alteration of the presynaptic $I_{Ca^{++}}$, as tested at various levels of step depolarization. Heat denaturation of synapsin I abolished its ability to produce this effect. Injection of CaM kinase II resulted in an increase in the amplitude and rate of rise of the postsynaptic potential and a decrease in the latency of the postsynaptic response, without a detectable change in the presynaptic $I_{Ca^{++}}$. The time-dependent effects observed for both proteins were consistent with the expected rates of diffusion of the molecules into the preterminal digit.

These preliminary results suggest that synapsin I and CaM kinase II play a definite role in synaptic transmission. They are most probably involved in regulating the availability of synaptic vesicles for neurotransmitter release in response to $I_{Ca^{++}}$ as phosphorylation of synapsin I by CaM kinase II is thought to detach synaptic vesicles from the synapsin I molecules.

Cerebellar field potentials evoked by optic nerve stimulation in the little skate, Raja erinacea. JAMES McGURK AND WERNER GRAF (The Rockefeller University, 1230 York Ave., New York, New York 10021).

The accessory optic system is one pathway by which visual information reaches the cerebellum. This system, which includes the nucleus of the Basal Optic Root in birds, amphibians, and fish, is considered to have a role in gaze stabilization and optokinetic nystagmus. As part of a study on visual-vestibular interaction among phylogenetically diverse animals we examined the physiological properties of the accessory optic system in elasmobranchs.

Field potentials evoked by optic nerve stimulation were recorded in the cerebellum of immobilized little skates, *Raja erinacea.* Recordings were made while the electrode was continuously advanced through the cerebellar cortex, and records were taken at 500 μm intervals. Stimulus parameters were 10 V pulses (threshold 2–3 V) of 100 μs duration at 0.5 to 2 Hz. General anesthesia with tricaine methanesulfonate (MS222) abolished all responses. The field potentials were found in a large region of the contralateral anterior lobe of the corpus cerebelli, in particular in the caudal two-thirds of the anterior lobe extending from the midline approximately one-half of the distance to the lateral edge. The field potentials were strictly

contralateral to the stimulated nerve and rostral to the antero-posterior fissure. Depth profiles from the area of maximal response revealed that the field extended from the surface to 2.5 mm beneath. At the surface the evoked field consisted of a positivity followed by a small negativity. At a depth of 0.5 and 1.0 mm the positivity was reduced while the negativity increased. The positive potential reversed completely to a negativity at a depth of 1.3 mm. The negativity reached a peak of 250 μV at 2.0 mm. The field potential occurred with a latency of 12 ms after the stimulus. Our data demonstrate a fairly localized rather than generalized input to the cerebellum from optic nerve stimulation in this elasmobranch species.

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Neomycin and other aminoglycoside antibiotics depress a light scattering change associated with secretion from nerve terminals of the mammalian neurohypophysis. T. D. PARSONS, A. L. OBAID, AND B. M. SALZBERG (University of Pennsylvania).

Light scattering changes recorded from the terminals of the neurohypophysis of the CD-1 mouse monitor events associated with secretion (Obaid *et al.* 1983, *Biol. Bull.* **165:** 530) and provide a tool for the investigation of agents that effect neurotransmitter release. The aminoglycosides are a class of antibiotics that may exhibit ototoxicity and neuromuscular blockade as clinical side effects. Animal studies of the neuromuscular junction have shown that these drugs depress evoked transmitter release by competing with $[Ca^{++}]_o$ (Fiekers 1983, *J. Pharmacol. Exp. Ther.* **255:** 487).

Low concentrations of aminoglycoside antibiotics depressed the magnitude of this light scattering signal. A sixty percent decrease was observed following an 11 minute exposure of the preparation to a 220 μM concentration of neomycin. The effect was slowly reversed by washing with normal Ringer's solution. The light scattering signal responded to neomycin in a dose-dependent manner. A Lineweaver-Burke analysis indicated that neomycin (22 μM) behaves as a competitive inhibitor of $[Ca^{++}]_o$ when calcium was varied from 0.5 mM to 10 mM.

Another aminoglycoside antibiotic, gentamicin, which is commonly employed in tissue culture media at 100 μg/ml, also depressed the intrinsic optical change. This concentration was sufficient to decrease the light scattering signal by nearly 40% after 11 minutes exposure, and could also be reversed. The apparent inhibition of neuropeptide secretion by gentamicin may be pertinent to studies involving primary neuronal cultures, and particularly *in vitro* synapse formation.

The effects of aminoglycoside antibiotics on an intrinsic optical change correlated with secretion at vertebrate nerve terminals provide additional evidence that these agents act presynaptically and that they act as competitive antagonists of $[Ca^{++}]_o$. Moreover, the compatibility of the results reported here with data obtained from the neuromuscular junction reinforce the interpretation that the light scattering changes observed in the mouse neurohypophysis reflect processes closely related to neuropeptide release.

We are grateful to Dr. David M. J. Quastel for directing our attention to effects of neomycin. Supported by USPHS grant NS 16824 and a VMSTP fellowship to TDP.

A rise in intracellular calcium is necessary and perhaps sufficient for photoreceptor excitation and adaptation by inositol 1,4,5-trisphosphate. RICHARD PAYNE, ALAN FEIN, AND D. WESLEY CORSON (Marine Biological Laboratory).

Injections of 10–40 pl of calcium buffer solution into *Limulus* ventral photoreceptors reduced the area under the inward current generated by a 1-4 pl injection of 100 μM inositol 1,4,5 trisphosphate ($InsP_3$) dissolved in stock solution (0.1 M potassium aspartate, 0.01 M HEPES, pH 7) to 2 $\pm$3% of control values, and also blocked subsequent adaptation of the light-response by $InsP_3$. After injection of calcium buffer, responses of the same cells to 10 ms light flashes were slowed and diminished in amplitude. However their area increased to 157 $\pm$81% of control values. The calcium buffer solution contained 0.02 M Ca, 0.1 M K_2EGTA, 0.5 M MOPS, pH 7, pCa 7. Omission of calcium or MOPS did not alter the results of injection. Control injections of stock solution resulted in a small reduction in the $InsP_3$- and light-response areas to 78 $\pm$23% and 66 $\pm$19% of control values respectively. The large reduction in the $Insp_3$-response area after injection of calcium buffers suggests that a rise in intracellular free calcium concentration (Ca_i) is necessary for the actions of $InsP_3$. Pulse-pressure injection of 1 mM or 2 mM calcium in stock solution causes rapid inward currents of 10–30 nA lasting 1–2 s. These Ca-induced currents appear to have the same reversal potential as the light response and can only be elicited by injection into the light-sensitive lobe of the photoreceptor. The inward current is followed by adaptation of the light- and $InsP_3$-response. Both the inward current and the adaptation produced by Ca are abolished by injection of calcium buffer. Rapid injection of calcium therefore mimics some of the actions of $InsP_3$.

$InsP_3$ may excite and adapt the photoreceptor solely by causing a rise in Ca_i. Light, however, must excite the cell by an additional pathway to account for the persistence of light responses after injection of calcium buffer.

A circadian clock increases the gain of photoreceptor cells of the Limulus *lateral eye.* GEORGE H. RENNINGER, EHUD KAPLAN, AND ROBERT B. BARLOW, JR. (Marine Biological Laboratory).

At night a circadian clock in the *Limulus* brain transmits neural activity to the lateral compound eyes increasing their sensitivity to light. Optic nerve activity recorded from the axons of eccentric cells (interneurons which receive input from the photoreceptor cells) indicates that at night the clock (1) decreases spontaneous activity in the dark, (2) decreases the threshold for the light response, and (3) increases the response to light at all intensities (Barlow *et al.* 1977, *Science* **197:** 86–89). The decrease in threshold and part of the increase of the light response, effects (2) and (3), can be attributed to an increase in the quantum catch of photoreceptors caused by circadian changes in the structure of the retinal cells (Barlow *et al.* 1980, *Science* **210:** 1037–1039). The remaining increase in the light response at intermediate and high levels of illumination reflects an increase in gain (response/absorbed photon).

We report here that the increase in retinal gain originates in the photoreceptor cells themselves. Photoreceptor potentials were recorded from single retinular cells *in situ* while the animal remained securely fastened to a rigid platform located in an aquarium inside a light-tight shielded cage. At intermediate and high levels of illumination the elevated potentials recorded at night appear to result from a depolarization-dependent increase in photoreceptor gain together with the known increase in quantum catch.

At the onset of the circadian clock input to the retina, photoreceptor gain increases more rapidly than quantum catch. Mimicking the clock's input to the eye with optic nerve shock clearly separates the two mechanisms that increase the light response. The increase in quantum catch requires extended periods of nerve shock and can be accounted for by structural changes in the retinal cells. The increase in gain begins within seconds of shock onset and may result from the circadian modulation of ionic conductances in the photoreceptor cell membrane.

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Calcium oscillations in the neuropile of crab stomatogastric neurons detected with Arsenazo III. WILLIAM N. ROSS AND KATHERINE GRAUBARD (New York Medical College Valhalla, New York).

The membrane potentials of many neurons of the stomatogastric ganglion of the crab, *Cancer irroratus,* have a natural oscillation. This oscillation is correlated with synaptic transmission in these cells and with some of the behavior controlled by this ganglion. It is likely that these oscillations are also related to changes in intracellular calcium.

We examined the location, timing, and magnitude of calcium concentration changes in some of these cells using the indicator dye Arsenazo III. The preparation was mounted on the stage of a Zeiss Universal microscope and imaged onto a 10×10 array of photodiodes. Using a $25\times$ water-immersion lens, each element detected light coming through a $60\ \mu m \times 60\ \mu m$ area of the ganglion. Dye was iontophoresed into the cell body of an identified neuron and allowed to diffuse throughout the cell. For each diode element the increase in absorbance at 640 ± 30 nm during natural or stimulated changes in potential was used to indicate an increase in intracellular calcium concentration at that position. After the experiment the cell was filled with Lucifer Yellow. The resulting cell shape was then superimposed on a map of the photodiode array allowing identification of those regions of the cell which showed absorbance changes.

In response to somatic stimulation, absorbance signals were detected over the soma, axon, and neuropile regions of most neurons. In two pyloric neurons oscillating changes were detected during spontaneous oscillations of the cells. These spontaneous absorbance changes were detected from the neuropile, but not the soma. There was a delay of 75–100 ms between the peak of the membrane potential and the peak of the absorbance change, corresponding to a phase shift of 30–50 degrees. In one of these cells the oscillating absorbance changes could be detected without averaging. We estimate that in this case the swing in calcium concentration may have been as large as 0.1 μM.

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Decline in calcium cooperativity as a mechanism of facilitation at the squid giant synapse. E. F. STANLEY (Marine Biological Laboratory).

Calcium entry in the nerve terminal is the link between the action potential (AP) and the release of transmitter. It has been suggested that four Ca^{2+} ions are required for the release of each transmitter quantum at the frog neuromuscular junction (Dodge and Rahamimott 1967, *J. Physiol.* **193:** 419–432). This conclusion is based on the plot of endplate potential (EPP) amplitude to external Ca^{2+} concentration at low non-saturating Ca^{2+} levels, which has a power function of four. At the squid giant synapse, however, there has

been large disparity in the reported power functions ranging from 1 to 3.5, probably due to the diffusion barrier that impedes the equilibration of external Ca^{2+} solutions in this preparation. In the present study the relation of EPP amplitude to Ca^{2+} has been re-examined, taking advantage of the ready access of substances to the synapse by aortic perfusion (Stanley and Adelman 1984, *Biol. Bull.* **167**: 467–476). In addition it is suggested that changes in Ca^{2+} cooperativity may be a mechanism of transmitter release modulation at this synapse.

EPPs were recorded at a stimulus frequency of <0.01 Hz during perfusion of the ganglion with artificial sea water containing 2, 3, 4, 5, 6, 8 or 10 m*M* Ca^{2+}, adjusted for osmolarity with NaCl. A plot of EPP amplitude against Ca^{2+} on logarithmic coordinates yielded a power function of 3.9 ± (S.E.) 0.1, n = 19. At higher stimulus frequencies the power function declined so that at 10 Hz it was 2.3 ± 0.3, n = 6 and 50 Hz 1.7 ± 0.4, n = 6. No significant change in the presynaptic AP was detected.

Thus, the power function, and hence Ca^{2+} cooperativity, is four at this synapse as at the neuromuscular junction. The decline of cooperativity at higher stimulus frequencies suggests a simple model that may explain facilitation at this synapse, based on the idea that dissociation of Ca^{2+} from its binding to the transmitter release site is slow. Thus, if the Ca^{2+} that enters and binds to a release site after one impulse does not fully dissociate before the next impulse, fewer additional Ca^{2+} ions entering during the second impulse need bind to reach the four necessary for release site activation.

Acetylcholine modulated potassium channels in the vestibular hair cell of the toadfish, Opsanus tau. A. STEINACKER (Washington University School of Medicine).

The vertebrate vestibular hair cell tranduces its ciliary motion into transmitter release resulting in firing of action potentials in an afferent fiber. This information transfer is modulated by an efferent synapse on the hair cell and/or on the afferent fiber. The transmitter at this efferent synapse and its effect on transmitter release from the hair cell is unresolved, although the presence of acetylcholine (ACh) has been demonstrated by immunohistochemical methods. The following experiments were designed to test the effect of ACh on hair cell conductances at the single channel level using patch clamp technology. Hair cells were digested from the toadfish saccule using a 30 minute papain incubation (0.5 mg/ml) and gentle trituration. The cells adhered to uncoated plastic petri dishes in a marine teleost Ringer. Data was gathered from cell attached patches with 200 m*M* KCl in the pipette. The pipette was held at sustained levels of voltage and stepped to new levels to exclude voltage inactivated channels. The most obvious signal using this paradigm was a high conductance channel with a reversal potential around 0 to +20 mV in a cell attached patch and 0 mV in a cell detached patch with symmetrical KCl or K^+ glutamate in the bath. The opening rate is voltage sensitive, with increases with both positive and negative steps away from zero holding potential. The opening rate at zero holding potential (cell resting potential) is low (1–5/s). Following the application of ACh (10^{-6} *M*) in micro drops near the cell, the opening rate of this channel increased dramatically to values 3–5 times control levels. There was no change in single channel conductance or reversal potential. ACh applied to the inside of an inside out patch had no effect. ACh thus appears to increase the opening rate of a channel with characteristics of a potassium channel and does this when the ACh receptor and the channel are at different sites. Thus, the channel and receptor do not appear to be linked at the same site and an intracellular mediator of the effect is likely. The fact that the reversal potential for this channel sits near the resting potential of the cell makes it an ideal candidate for modulation of transducer currents, both in the on and off direction.

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Morphology and physiology of the giant interneurons of the hermit crab Pagurus pollicaris. PHILIP J. STEPHENS (Villanova University).

Two pairs of giant interneurons provoke escape tail-flips in crayfish and lobster. In hermit crabs, however, only one pair of interneurons with axons runs the length of the animal. These giant interneurons produce a rapid tail flexion and a withdrawal of the hermit crab into its shell. This study examines the physiology and morphology of this pair of neurons.

Iontophoretic injection of cobalt ions into the axons of the giant interneurons through a microelectrode, revealed that the cell bodies lie on either side of the mid-line in the anterior region of the "brain." The axon travels caudally from the cell and gives off an extensive dendritic field. It then crosses the mid-line, gives off a major branch into the lateral portion of the brain, and travels caudally to exit in the connective which is contralateral to the soma. No branching of the axon was observed in the thoracic and abdominal ganglia.

In some preparations, an action potential in one interneuron elicited a spike in the other. Transmission was bi-directional and lesion experiments indicate that the synapse is situated in the brain. There was no

dye (Lucifer yellow)-coupling between the two interneurons, and there was a time delay in transmission of about 0.4 ms. Synaptic transmission was Ca^{++} dependent, was not effected by dinitrophenol, and could be fatigued so that the response in the post-synaptic cell changed from an action potential to an excitatory post-synaptic potential. Therefore, the synapse between the two giant interneurons seems to be chemical.

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Chemosensitivity to nicotine in the crayfish slowly adapting stretch receptor. ROBERT F. TAYLOR (Department of Physiology, University of Kentucky, Lexington).

Evidence is increasing that mechanoreceptors in both vertebrates and invertebrates are capable of responding to chemical as well as mechanical stimuli. These experiments studied the effect of nicotine, a known neural stimulant in laboratory mammals and man, on the slowly adapting stretch receptor of the crayfish *Procambarus clarkii*. Crayfish muscle is not directly sensitive to nicotine or acetylcholine and therefore permits testing of any direct chemical effect of nicotine on the stretch receptor.

The crayfish tail was removed from the thorax and pinned out on wax in a petri dish. Musculature was dissected leaving the stretch receptors in the second or third abdominal segments intact. The afferent nerve of a single slowly adapting stretch receptor was monitored with a suction electrode, using appropriate nerve recording methods. A thread was attached to the telson and to force transducer in order to measure tension in the tail. The preparation was bathed in buffered van Harreveld's solution and kept at 18°C. All drugs were prepared in van Harreveld's solution and administered directly into the bath.

Under conditions of isotension, nicotine (0.07 μM) increased receptor activity four to five fold. This response was abolished by treatment with hexamethonium (690 μM). Desensitization was not a possible explanation for this effect as the addition of more nicotine to the bath resulted in a similar four to five fold increase in activity. In a second group of animals, sensitivity curves were generated by plotting step changes in tension against nerve activity. All plots were linear ($r > 0.93$) and only nicotine significantly increased the slope of the response. Hexamethonium was sufficient to block the effect on receptor sensitivity to changes in tension.

These results demonstrate that the sensitivity of a mechanoreceptor can be dramatically altered by chemical interaction with nicotinic receptors located somewhere on the receptor membrane.

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Chemoreceptor adaptation: responses of glutamate cells to repeated stimulation. RAINER VOIGT AND JELLE ATEMA (Boston University Marine Program, Marine Biological Laboratory).

The chemoreceptor cells on the walking legs of the lobster, *Homarus americanus*, remain narrowly tuned even at high concentrations of amino acids. Glutamate best cells are most prominent and seem to form a homogeneous, mostly narrowly tuned spectral population. Theoretically, these cells can provide more unambiguous information on stimulus concentration differences than broadly tuned cells. In order to understand their role in signal detection, we began to characterize one of their dynamic response properties (here adaptation) to standard glutamate pulses by varying the interpulse interval.

Single glutamate cells were identified electrophysiologically with a 3×10^{-6} M glutamate search stimulus. A series (5–10) of 1-second pulses of 3×10^{-4} M glutamate was applied with one of four pulse intervals; 20, 40, 80, or 160 s. Responses were measured as the number of spikes in 100 ms time bins; most response durations did not exceed 15 s.

Of 30 single cells 70% gave a phasic-tonic response; its frequency time profile resembled the stimulus profile. These cells may represent sensitive glutamate cells which do not adapt for the duration of the stimulus (no *intra*pulse adaptation). When stimulated in intervals longer than 20 s their response (both total number of spikes and the duration of the response) varied only slightly (<20%) over 5 successive stimulations; 20 s pulse intervals caused a systematically decreasing response to less than 50% of the first response (*inter*pulse adaptation). Response variability was greatest in the tail of the response (after the first 2–3 s).

Similar adaptation was seen in cells responding with double bursts or mostly tonic responses. However, a third group of cells gave only short responses (<3 s). Such cells may be less sensitive or exhibit rapid *intra*pulse adaptation; they did not show *interpulse* adaptation even at 10 s pulse intervals.

These results reveal the possibility that the spectrally consistent glutamate cells separate into subgroups defined by different dynamic response properties.

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Characterization of the sea robin Mauthner cell. STEVEN J. ZOTTOLI (Williams College) AND MARK A. AGOSTINI.

The goldfish Mauthner cell (M-cell) provides an accessible system in which to examine factors which influence the regenerative capacity of an identifiable vertebrate CNS neuron. To identify possible interspecific differences with respect to regeneration, we examined the sea robin (*Prionotus carolinus*). As a necessary prelude to this endeavor, this report characterizes basic electrophysiological and morphological properties of the sea robin Mauthner cell.

Intracellular recordings were made with KCl electrodes. Lucifer yellow filled electrodes were used for intracellular staining. Lightly anesthetized (0.007% MS-222) and unanesthetized fish were used in these experiments. M-axons were penetrated just rostral to the commisura infima and criteria for their identification included visualization, sound-evoked responses, depth (30–60 μm) below the medullary surface and the elicitation of head and body responses following intra-axonal stimulation.

The sea robin M-cell has similar morphology to that of the goldfish except that the axon is smaller and the soma has more extensive medial dendrites. In contrast to the goldfish, antidromic activation could not be elicited throughout the entire length of the spinal cord. Subthreshold stimulation resulted in an EPSP of 2.9 $\pm$ 1.1 mV in amplitude. Threshold stimulation resulted in an all-or-none spike occurring 0.9 ms $\pm$0.34 earlier than the EPSP. M-axon conduction velocity was 26 m/s (n = 2; 20°C) using two point penetrations with the recording electrode. Intracellular stimulation of the M-axon elicits movements of the jaw, eyes, operculi, fins, and trunk and tail musculature. EMG recordings of the trunk musculature revealed both ipsi- and contralateral responses. The ipsilateral response was both larger and occurred earlier than that recorded contralaterally.

The results of this study demonstrate the feasibility of using the sea robin Mauthner cell as a model for regeneration. Its morphological and electrophysiological similarity to the goldfish M-cell and the presence of stereotypical M-cell responses ensures unambiguous identification. With respect to concurrent studies using posterior lateral line implants to facilitate M-cell regeneration in the sea robin, the smaller axon diameter and the expected reduction in axonal retraction distance may increase the chance that a lesioned M-axon stays more closely apposed to the implant allowing for contact and insertion of its sprouts.

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PARASITOLOGY AND PATHOLOGY

Expression of malaria antigens in Escherichia coli: *cloning of* Plasmodium vivax *DNA cut with mung bean nuclease.* PETER H. DAVID (Institut Pasteur, Paris), KAMINI N. MENDIS, RABIA HUSSAIN, THOMAS F. MCCUTCHAN, AND DYANN F. WIRTH.

Little is known about the antigenic make-up of *Plasmodium vivax*, the second most important human malarial parasite after *Plasmodium falciparum*. This is due in part to the impossibility of maintaining *P. vivax* in culture. To obtain *P. vivax* antigens *in vitro* and explore the organization of the plasmodium genome, a *P. vivax* genomic DNA library was constructed in *E. coli*. DNA was extracted from parasites obtained from a single patient in Sri Lanka. The high degree of purity of the DNA preparation was assessed by comparative restriction enzyme digestion analysis of human and *P. vivax* DNA. Plasmodium DNA was then cut with mung bean nuclease; in formamide, this enzyme was found to cut *P. falciparum* DNA at sites located before and after genes, not in coding regions (McCutchan *et al.* 1984, *Science* **225**: 625–628). Fragments of *P. vivax* DNA obtained from mung bean nuclease digestion in 35%, 40%, and 45% formamide were pooled and ligated to the expression vector PUC 12 in the β-galactosidase gene cut with restriction enzyme SMA I. The vector was then used to transform *E. coli*, strain TB1. A partial library of 30,000 transformed bacterial colonies was obtained with 1 μg of vector DNA. Antibody-screening of bacterial colonies was performed with hyperimmune human sera and with monospecific rabbit immune sera directed against purified plasmodium antigens, followed by radiolabelled protein A. The first screening of 200 colonies revealed 6 colonies which were specifically recognized by antibody, this leading to the conclusion that transformed *E. coli* may be expressing *P. vivax* antigens.

Variation in growth of post-metamorphic Aplysia californica. MICHAEL J. GOOD, THOMAS R. CAPO, AND LLOYD NADEAU (Howard Hughes Medical Institute; Marine Biological Laboratory).

Biomedical use of *Aplysia californica* as a model in neurobiological and behavioral research has prompted the need for mass culture techniques that reliably supply animals of known parentage, age, and

environmental history. Large numbers of post-metamorphic animals can now be obtained in natural sea water using present culture techniques. During the period of February to August, 1984, we successfully raised 1000 animals to a size greater than 5 g wet weight. Considerable variation within and between these sibling groups was observed. Subsequently, experiments were designed to investigate this variation as a function of stocking density.

Sibling groups were maintained at densities of 5, 10, 20, and 50 animals per 16 liter flow-through tanks with approximate flow-rates of 0.5 l/min. Tanks were cleaned daily and animals were fed *ad libitum* *Ulva lactuca, Gracilaria* sp., or *Agardhiella subulata.* Results indicate a negative correlation between density and final (1) mean wet weight (g) and (2) mean growth rate (g/day). At densities of 5 and 50 animals per tank, final mean wet weights were 29.6 ± 8.4 g and 10.3 ± 6.6 g, while mean growth rates were 1.4 and .55 g/day, respectively. These data suggest that density is an important parameter effecting post-metamorphic growth of *Aplysia californica.*

A protective monoclonal antibody to S. mansoni *induces both anti-idiotypic and idiotypic monoclonal antibodies as well as anti-idiotypic T cells.* D. HARN, L. LEUM, G. JEFFERS, AND D. COLLEY (Harvard Medical School).

Chronic *Schistosomiasis mansoni* results in immunoregulation of the host-patient response to the schistosome egg. This regulation may also involve aspects of the protective immune response to challenge. In an earlier study, we produced an anti-egg monoclonal antibody (E.1) which binds to surface epitopes on schistosomula and is partially protective *in vivo* (Harn *et al.* 1984, *J. Exp. Med.* **159:** 1371–1387). We used this anti-egg monoclonal antibody to study the anti-idiotypic aspect of immunoregulation in *S. mansoni.*

Syngeneic mice were primed and boosted by intravenous and intraperitoneal injection of pure E.1. Mice were sacrificed four days after the final boost and their spleen cells were used for the production of B cell hybridomas or T cell lines.

Spleen cells cultured for anti-idiotypic T cells were pulsed with interleukin-2 and E.1. The viability counts showed a 600% increase at one week, followed by a 300% increase at the second week. Aliquots of putative anti-idiotypic T cells have been frozen for further testing.

The B cell fusion gave rise to 145 immunoglobulin secreting hybridomas. Twenty (13%) of these hybrids showed anti-idiotypic activity for E.1. The hybrids reduced E.1 binding to soluble egg antigen (SEA) by 62–82%. The hybrids were tested for idiotypic activity by assaying for binding to SEA. Five hybrids (3.5%) bound to SEA as well as to the surface membranes of living schistosomula. Three of the five idiotypic hybrids also immunoprecipitated the same molecular weight antigen as E.1.

Thus, anti-idiotypic and idiotypic monoclonal antibodies as well as anti-idiotypic T cell lines have been produced from mice immunized with protective monoclonal antibody E.1. This shows that the complete anti-idiotypic loop occurred in the same animal.

Larval health of Hermissenda *in mass culture.* MICHAEL JOHNSON, THOMAS CAPO, ALAN M. KUZIRIAN, LOUIS LEIBOVITZ, HEATHER URQUHART, AND DANIEL L. ALKON (Marine Biological Laboratory: Laboratory of Biophysics, NINCDS-NIH and Laboratory for Marine Animal Health).

Preliminary observations were made on the larval health of the nudibranch *Hermissenda crassicornis* in mass culture. Developmental and pathologic changes were observed, and used to evaluate suitability of egg masses and larvae for rearing. Egg masses were taken soon after oviposition from wild adults maintained under laboratory conditions. Egg masses were incubated at 16°C ± 2 and aerated in a 1 liter flask of millipore prefiltered sea water. Emerging larvae were reared in 2 liter flasks, using two different antibiotic regimes plus a control of millipore filtered sea water. Chloramphenicol and penicillin/streptomycin were used at concentrations of 2.5 mg/l, 60 and 50 μg/ml, respectively. The larvae were fed a diet of algae consisting of T-*Isochrysis* and *Chloromonas* either alone or in combination (5×10^3 cells/ml). Observations and shell lengths were taken every 3–4 days and selected samples were preserved for histological procedures including SEM. Preliminary results show no significant difference in the growth of the shell between cultures with chloramphenicol, penicillin/streptomycin, and the control through eleven days.

Pathologic changes were observed in various larval organ systems and shell. Changes seen in the shell consisted of shell deformities and numerous uneven growth bands, especially on slow growing larvae. Also noted were varying degrees of calcification between recently hatched larvae of the same egg mass. Pathology seen in the organ systems was often associated with inanition. The visceral mass and mantle were markedly contracted away from the shell. In non-feeding larvae, the digestive gland was often atrophied and green as compared to the uniform reddish brown of healthy larvae. Details of other viscera were often obscured and unclear in these same larvae. Larvae with bacteria on the velum and epiphytes on the shell and velum were also observed in poor growing cultures.

One of the more perplexing observations made was in the development of the embryos within the egg strand. There was a marked degree of variability in larval development within the same egg strand and occasionally within the same egg capsule. The variability in development occurred in different sections within the same egg strand. Variability within these sections was also non-uniform, as was the variability of development between different egg masses from different adults. There was, however, little if any developmental variability between the proximal and distal portions of an individual egg strand. Whether the variability in development occurs naturally, is related to age, size, health, nutrition, or environmental experiences of the larvae or adult, is a subject for future research.

M. Johnson was funded by a fellowship from the Charles Ulrich and Josephine Bay Foundation.

Amoebiasis of larval sea hares (Aplysia californica). LOUIS LEIBOVITZ (Marine Biological Laboratory), JOHN A. PAIGE, AND JOSEPH BIDWELL.

Sea hares (Aplysiidae) are estuarine opisthobranch molluscs with world-wide distribution. They are used extensively as biomedical research models in neurobiological, behavioral, reproductive, and other biological studies. The need for a continuous supply of healthy standardized animals of known genetic stocks reared in a controlled environment has encouraged laboratory culture of aplysiids in chemically defined media (synthetic sea water) throughout its complicated life cycle. During development of the above cultural methods, repeated epizootics of amoebiasis in pre-metamorphoric larval sea hares occurred. First signs of the disease were attachment of amoebae to the surface of the larval shell, and progressive movement towards the exposed soft tissues of the velum at the opercular region. The amoebae phagocytized the soft velar tissues of velum, rapidly underwent binary fission, and extended inwardly into the body and mantle cavities, eventually consuming all of the noncalcified tissues of the larvae. The cavity of the shell then became completely filled with amoebae which later escaped into the cultural media and became attached to other larvae. Larval mortality approached 100 percent within a 7 day period.

The results of light and electron microscopic examination of fresh specimens and fixed stained histological sections were presented.

The amoeba, from fresh material measured 20 to 50 μm in length, conformed to members of the marine class Conopodina, being finger-like in shape when fully extended with unbranched lobose pseudopodia. Other structures observed were a uroid, granules, and cysts containing phagocytized tissue. The nucleus and amphosome conformed to those described for the family Paramoebidae. The amoeba has been maintained in natural sea water. Studies are underway to further characterize the amoeba and the disease produced. This is the first report of a specific highly fatal form of amoebiasis in molluscs and a description of the etiologic agent.

This study is supported in part by a grant from the National Institutes of Health (P40-RR1333-03) and the Howard Hughes Medical Institute.

Digestion of tubulin genes from Leishmania enriettii *with mung bean nuclease.* KIM LEE SIM, SCOTT LANDFEAR, AND DYANN WIRTH (Harvard School of Public Health).

We are studying the structure and the regulated expression of the tandemly repeated tubulin genes from the parasitic protozoan *Leishmania enriettii.* Recent work (McCutchen *et al.* 1984, *Science* **225**: 625–628) on another parasitic protozoan, *Plasmodium falciparum,* has shown that the enzyme mung bean nuclease introduces very discrete cuts in front of genes and after genes, but not within the coding region of a gene. These discrete extragenic cuts occur for both whole chromosomal DNA and for cloned fragments of genomic DNA. These results imply that some secondary structural feature of DNA, which is fortuitously recognized by mung bean nuclease, occurs before and after genes in *Plasmodium.* The widespread occurrence of this structure between genes in *Plasmodium* suggests it possesses some biologically important function.

To determine whether *Leishmania* genes also possess similar mung bean sensitive sites in the intergenic regions, we have digested with mung bean nuclease both chromosomal DNA and the alpha-tubulin genomic clone pLT1. We mapped the position of the mung bean digestion sites using Southern blot analysis and have shown that they occur between the tandemly repeated alpha-tubulin genes. Specifically, the 2 kilobase genomic insert of the alpha-tubulin clone is digested by mung bean nuclease to give two fragments of size 1450 and 600 base pairs. Since this clone contains the 5′ end of one tubulin gene and the 3′ end of the adjacent tubulin gene, with the intergenic region at a position about 1450 base pairs from one end and 600 base pairs from the other end, this result suggests that mung bean nuclease cuts at a discrete site between adjacent copies of alpha-tubulin genes. Chromosomal DNA digested with mung bean nuclease also generates these same 1450 and 600 base pair fragments, confirming the results obtained with the cloned genomic DNA.

PHYSIOLOGY

An unusual form of alpha-2-macroglobulin isolated from the plasma of Limulus polyphemus. PETER B. ARMSTRONG (University of California, Davis) AND JAMES P. QUIGLEY.

The alpha-2-macroglobulins (α_2M) are a family of high molecular weight proteinase-binding proteins that display a variety of unique characteristics that distinguish them unambiguously from other proteinase inhibitors. (1) α_2M shows an unusually broad spectrum of activity, inhibiting proteinases from each of the four classes of proteinases. (2) Although the ability of bound proteinases to hydrolyze macromolecular substrates is inhibited, the enzymatically active site is intact and low molecular weight amide and ester substrates are hydrolyzed. (3) The bound proteinase molecules are shielded from high molecular weight active site inhibitors. The thiol ester class of α_2M is characterized by sensitivity to low molecular weight primary amines such as methylamine, whereas the ovostatin class of α_2M is insensitive. α_2M is present in the plasma of all classes of vertebrates. We have demonstrated, based on the characteristics listed above, an α_2M homologue of the thiol ester class in the plasma of the horseshoe crab *Limulus polyphemus*. Purified *Limulus* α_2M has been compared with human α_2M. The subunit molecular weight determined by SDS-polyacrylamide gel electrophoresis (reducing conditions) is 182×10^3 for human α_2M and 187×10^3 for *Limulus* α_2M. Native human α_2M is a tetramer ($M_r = 728 \times 10^3$). The α_2M of fish has been reported to be a dimer ($M_r = 360 \times 10^3$). In contrast, native *Limulus* α_2M appears to be a trimer. By gel filtration on Sephacryl S-300 resin, *Limulus* α_2M shows a molecular weight of 571×10^3. It elutes at a molecular weight position lower than the marker protein thyroglobulin ($M_r = 669 \times 10^3$) and higher than ferritin ($M_r = 443 \times 10^3$), whereas human α_2M run on the same column elutes at a molecular weight position higher than thyroglobulin. Gel filtration chromatography has been conducted both in 0.1 *M* Citrate, pH 6.5 and in 0.5 *M* NaCl-0.025 *M* Tris, pH 7.75. The subunits of *Limulus* α_2M appear to be covalently linked because the molecule runs at a higher molecular weight position than dimeric fibronectin ($M_r = 440 \times 10^3$) and the dimeric form of human α_2M ($M_r = 364 \times 10^3$) by SDS-polyacrylamide gel electrophoresis (non reducing conditions). Under these conditions, as noted above, human α_2M dissociates from the native tetrameric form to the dimeric form.

This study was supported by NSF grant #PCM 80-24181.

Anadara ovalis *hemoglobins: distinct dissociation and ligand binding characteristics.* THOMAS A. BORGESE (Lehman College-CUNY), JOHN P. HARRINGTON, AND RONALD L. NAGEL.

Electrophoresis of hemoglobins from pooled clam red cells reveals a major (Hb_{ma}, anodal) and a minor (Hb_{mi}, cathodal) hemoglobin whose molecular weights (m.w.) determined osmometrically are 56,200 and 29,900, respectively. Globin chain analysis by HPLC indicates that Hb_{mi} is a homodimer (two identical chains or, alternatively, two similar chains with a limited number of neutral amino acid differences) and Hb_{ma}, a heterotetramer with three chains, none of which is identical to the dimer. Chain analysis of individual clams provided evidence of polymorphism. Oxygen equilibria studies show Hb_{ma} P_{50} to be concentration but not pH dependent, unaffected by addition of ATP, and a Hill coefficient (n) ranging from 2–2.4. In contrast, Hb_{mi} has a higher oxygen affinity, is not concentration dependent, is unaffected by ATP or pH, and has n values of approximately 1.4.

Anadara ovalis hemoglobins are less resistant than human Hb to isothermal denaturation by urea. The order of decreasing resistance is HbCO > HbO > HbMet for both Hb_{ma} and Hb_{mi}. The thermal denaturation times (50% denaturation at 50°C) for oxyHb (major), oxyHb (minor) and carbonmonoxyHb (major) are 9, 88, and 1000 minutes, respectively. The carbon monoxide ligand renders the tetramer considerably more thermostable.

Gel filtration indicates that NaI, propylurea, and guanidine-HCl, at concentrations known to dissociate fish and human hemoglobins (1.0 *M*), fail to dissociate Hb_{ma} or Hb_{mi}. This increased resistance to dissociation may be related to particular amino acid residues at pertinent sub-unit interfaces. With the exception of a small polymer peak, both hemoglobins of *Anadara ovalis* are dissociated to 16,000 m.w. subunits on conversion to methemoglobin by the addition of potassium ferricyanide. Both phenomena, polymerization and monomerization, are completely inhibited by 0.1 *M* β-mercaptoethanol. We suspect that methemoglobin formation brings two intra-subunit -SH groups close enough to generate an S-S bond. This weakens the inter-subunit attractions and causes dissociation into monomers.

This work was supported by PSC-CUNY Grants #RF 62148 and 64123, The University of Alaska Faculty Research Grant, and NIH Grant #HL 621016.

The scavenging of ascorbic acid and semiquinone free radicals by normal and transformed cells. PETER GASCOYNE (Marine Biological Laboratory), FREDERICK BECKER, CHIU-NAN LAI, JANE MCLAUGHLIN, RONALD PETHIG, AND ALBERT SZENT-GYÖRGYI.

The ability of Ehrlich ascites tumor cells to scavenge a long-lived population of 2,6-dimethoxy-p-benzosemiquinone and ascorbyl free radicals has been demonstrated by electron spin resonance spectroscopy (Pethig *et al.* 1984, *Proc. Natl. Acad. Sci. USA* **81**: 2088–2091). We extended these studies to investigate the radical scavenging ability of three cultured cell lines derived from rat kidney, and normal and transformed Chinese hamster ovary (CHO) cells.

Cells designated 6M2 were cloned from normal rat kidney (NRK) cells that had been infected with a temperature sensitive mutant of Moloney murine sarcoma virus and exhibited a transformed phenotype at 33°C but a normal phenotype at 39°C. A second transformed cell line, designated 54-5A4, was a clone of 6M2 no longer possessing a temperature sensitive phenotype. When grown in dibutyryl cyclic AMP, CHO cells exhibited a normal phenotype, but in its absence they showed a transformed phenotype.

The radical scavenging ability of NRK cells was the weakest, that of 6M2 cells in their transformed state was four times stronger than NRK, and that of 54-5A4 cells was 10 times greater than NRK. 6M2 cells of normal phenotype exhibited similar scavenging characteristics to NRK. CHO cells of transformed phenotype exhibited a scavenging ability some four times greater than the normal CHO cells. It has been concluded that the ability of the various cell lines studied to quench ascorbyl and semiquinone free radicals directly parallels their state of transformation.

This work is supported by the National Foundation for Cancer Research and in part by the Agnes and Thomas Williams Fund for Studies in Tumor Cell Surfaces.

Inhibition of a Soman- and DFP-detoxifying enzyme (DFPase) by the organophosphorus insecticide, Mipafox. FRANCIS C. G. HOSKIN (Illinois Institute of Technology).

There are at least two so-called DFPases (Hoskin *et al.* 1984, *Fundam. Appl. Toxicol.* 4, No. 2, Part 2: S165–S172). One, squid type DFPase, is narrowly distributed in cephalopod nerve, hepatopancreas, and saliva. It hydrolyzes the cholinesterase inhibitor DFP (diisopropyl phosphorofluoridate) much faster than the vastly more potent cholinesterase inhibitor Soman (1,2,2-trimethylpropyl methylphosphonofluoridate); it is easily purified by ammonium sulfate fractionation, has a molecular weight of 26,600, and is indifferent to Mn^{++}. The more widely distributed enzyme is—Mazur type DFPase (named in recognition of its discoverer)—hydrolyzes Soman much faster than DFP, has a much higher molecular weight, is ammonium sulfate labile, and is stimulated several-fold by Mn^{++}. In a search for natural substrates and physiological roles for these unusual enzymes, many compounds have been considered, especially those displaying properties in addition to the inhibition of the ChE's. One such, Mipafox (N,N'-diisopropylphosphordiamidofluoridate) showed considerable promise as an insecticide, inhibiting AChE at about 10^{-4} *M*, and ChE at about 10^{-7} *M*. However, from its earliest (and hence limited) use, Mipafox was implicated in a slow degeneration of nerve fibers caused by the inhibition of "neurotoxic esterase."

Now it is found that Mipafox is a potent, reversible, competitive inhibitor of Mazur type DFPase, but not of squid type DFPase, using either DFP or Soman as substrate. The K_i is approximately 1.7×10^{-5} *M* when the K_M for Mazur type DFPase with DFP as substrate is 1.7×10^{-3} *M*. In more immediate terms, 3×10^{-4} *M* Mipafox causes 90% inhibition of Mazur type DFPase, whereas 3×10^{-3} *M* Mipafox causes no inhibition of squid type DFPase, whether DFP or Soman is substrate, and these at 3×10^{-3} *M*. Mipafox itself is barely hydrolyzed by either enzyme (about 1% the rate of DFP). Neither enzyme is the "neurotoxic esterase."

This work was supported by a grant from the ARO.

Studies of the scavenging of anionic free radicals by Ehrlich ascites cells. RONALD PETHIG, PETER GASCOYNE, JANE MCLAUGHLIN, AND ALBERT SZENT-GYÖRGYI (Marine Biological Laboratory).

Earlier studies by Szent-Györgyi *et al.* (1983, *Biol. Bull.* **165**: 496) showed, as a result of the close matching of their mid-point redox potentials, a mixture of 2,6-dimethoxy-p-benzoquinone and sodium ascorbate at pH 7 provides a long-lived population of semiquinone and ascorbyl anionic free radicals. The lifetimes of these radicals in ascitic fluid were directly proportional to the concentration of viable Ehrlich ascites tumor cells present in the ascitic fluid.

Our recent studies showed that the rate of scavenging of these radicals by ascites cells is related to glycolysis, to the number of cell-surface sulfhydryl groups, and is increased by the action of neuraminidase in reducing cell-surface charge. By adopting the electrical double layer theories of colloidal chemistry and

taking measurements as a function of the ionic strength of the cell suspending medium, a value of -1.22 $\mu C \cdot cm^{-2}$ has been obtained for the charge density at the ascites cell surface. This charge density value is in good agreement with that obtained from cell electrophoresis measurements.

Homogenized ascites cells are not able to scavenge the free radicals, but the scavenging ability is restored by the addition of 0.25 m*M* NADH or NADPH to the homogenates. The blocking of $-SH$ groups by N-ethylmaleimide or heat denaturation results in a loss of this NADH-dependent scavenging activity. Fractionation of the homogenate by differential centrifugation has provided strong evidence for the scavenging activity not to be associated with the cell nucleus or mitochondria, and the indications that it is a cell membrane associated phenomena are in agreement with the earlier conclusions obtained from the studies on viable cells.

Mr. Richard Meany assisted with cell-line management and characterization, and the work is supported by the National Foundation for Cancer Research.

Stimulation of bioluminescence in fluid flow. GEO. T. REYNOLDS (Department of Physics, Princeton University), ALAN J. WALTON, AND DONALD M. ANDERSON.

Previous experiments studied the response of dinoflagellates in dilute suspensions to mechanical stimulation (Reynolds and Walton 1983, *Biol. Bull.* **165:** 522). These studies have been extended to include the effect of flow through capillaries, response to movement of spheres and ellipsoids, and response to flow set up by rotating bodies of various shapes. Velocities were adjusted to provide a wide range of Reynolds numbers. Physical parameters studied included velocity, acceleration, shear, and pressure.

Detailed data were obtained from film and video tape records of the output of an image intensifier. Results show that *Gonyaulax polyedra* does not respond to *constant:* shear fields; overpressures; reduced pressures; velocities; or accelerations. Variations in parameters causing stimulation have been recorded. In addition, surface effects and response to bubble formation were particularly pronounced.

We thank P. Botos, Jr. for the preparation of the mechanical components of the equipment, and David Kulis for the preparation of the cultures. This work was supported by DOE Contract DE-AC02-76EVO 3120, ONR Contract N0014-83-C-0234, and NSF Contract OCE-8208739.

An alpha-2-macroglobulin-like proteinase-binding activity in the blood of crustaceans. MICHAEL T. ROSSNER (Princeton University), PETER B. ARMSTRONG, AND JAMES P. QUIGLEY.

Alpha-2-macroglobulin (α_2M) is a high molecular weight proteinase-binding protein that forms a cage-like structure around bound molecules of trypsin and other proteinases. The bound trypsin is sterically prevented from hydrolyzing proteins but retains catalytic activity against low molecular weight amide and ester substrates. The bound enzyme is shielded by the α_2M from high molecular weight active site inhibitors such as soybean trypsin inhibitor (SBTI). The blood of various invertebrates was screened for an α_2M-like activity, *e.g.*, for the ability to protect the amide hydrolytic activity of trypsin from SBTI. *Limulus* plasma contains large amounts of this activity. Activity could not be detected in coelomic fluid from *Venus mercenaria, Arbacia punctulata,* and *Ciona intestinalis.* The blood of the decapods *Cancer borealis* and *Libinia emarginata* contained low but detectable amounts of an α_2M-like activity. Plasma from the crustaceans was capable of binding 2–20 μg of trypsin per ml. Two reactions can interfere with the α_2M assay: (1) low molecular weight endogenous active site inhibitors can enter the α_2M "cage" and inactivate bound trypsin, and (2) endogenous proteinases released or activated during bleeding inactivate plasma α_2M. The highest activity was observed in preparations in which blood was collected directly into saline containing trypsin, with lower quantities detected in blood collected into anticoagulants. Conventional preparations of serum were negative, presumably because endogenous proteinases released or activated during bleeding complexed the α_2M. *Libinia* α_2M was inactivated by methylamine (MA), indicating that it belongs to the thiol ester class of α_2M. The α_2M activity of *Cancer* whole plasma was insensitive to MA but showed partial sensitivity in preparations that has been partially purified by gel filtration chromatography. The molecular weights of *Cancer* and *Libinia* α_2M were determined by gel filtration on Sephacryl S-300 resin to be, respectively, 462×10^3 and 478×10^3.

This study was supported by N.S.F. Grant No. PCM 80-24181.

Stimulus-response coupling in marine sponge cells: studies employing fluorescent dyes to monitor effects of calcium pulses. LESLIE B. VOSSHALL, PHILIP DUNHAM, CYNTHIA BAYER, AND GERALD WEISSMANN (Marine Biological Laboratory).

Microciona prolifera cells, dissociated in Ca, Mg-free sea water with 2.5 m*M* EDTA, aggregate when exposed to Ca (>5 m*M*) and Ca ionophores (A 23187, ionomycin; 5–10 μM). We now report that extracellular

Ca is not required over the course (0–10 min) of aggregation: brief (1–3 s) pulses of Ca (5–20 m*M*; then EDTA, 10–40 m*M*) suffice. Aggregation was induced by A 23187 in excess EDTA, provided cells were "prepared" by pulse Ca. Since *Microciona* aggregation factor (MAF) rapidly dissociates in EDTA, and failed to aggregate pulsed cells in excess EDTA, it appeared possible that Ca ionophore provoked secretion of pre-formed MAF from intracellular compartments inaccessible to external EDTA. We therefore preloaded sponge cells with three fluorescent dyes which monitor aspects of stimulus-secretion coupling: (1) diS-C_3-(5), a carbocyanine dye presumed to report changes in surface membrane potential ($\Delta\Psi$); (2) 9-aminoacridine which presumably reports secretion from acid vesicles, and (3) chlortetracycline, presumed to report mobilization of membrane-associated Ca. Exposure of cells either to constant Ca (20 m*M*) or to pulse (1–3 s) Ca (20 m*M*; then EDTA, 40 m*M*) caused prompt decreases in fluorescence (*F*) of cells with diS-C_3-(5) and increases in *F* of cells with 9-aminoacridine. In contrast, while constant Ca provoked rapid decreases in *F* of cells with chlortetracycline, pulse Ca was without effect. Providing specificity of the probes, sponge aggregation is therefore accompanied by changes in $\Delta\Psi$, secretion from acid vacuoles, and mobilization of Ca from membrane sites sensitive to external EDTA. Moreover, inhibitors of stimulus-response coupling (*e.g.*, aspirin, Na salicylate, 5 m*M*; diclofenac, >100 μM) inhibit sponge aggregation. In contrast, like the endogenous mediator of inflammation, leukotriene B_4, trienoic alkyl catechols (urushiols) from poison ivy and poison oak provoke sponge aggregation. These studies suggest the utility of this marine model for analysis of stimulus-response coupling in inflammatory cells of higher species.

The exquisite squid lens: an alternative system of transparency and accommodation. SEYMOUR ZIGMAN, TERESA PAXHIA, AND WILLIAM WALDRON (University of Rochester School of Medicine and Dentistry, Rochester, New York).

Surprisingly, the squid (*Loligo pealei*) is a cephalopod whose ocular lens resembles that of vertebrates morphologically. Squid lenses are as transparent and magnify images as well as most marine vertebrates. Squid lenses have two segments: an anterior one-third derived from ectoderm of the outer optic vesicle wall, while the posterior two-thirds derive from its inner wall. Each segment grows by adding new layers of fiber cells concentrically, so that the oldest lens fibers are concentrated in the nuclear core while the youngest are present peripherally in the cortex. Lens weight increases linearly relative to body weight and length, and thus to aging. Scanning electron microscopy shows lens fiber cells to be irregularly cylindrical, with few interdigitations via tongue and groove connections. Fibers vary in size and orientation, and appear to be thicker in the lens nucleus than in the cortex.

Aggregated, water-insoluble proteins accumulate progressively in the nucleus of squid lenses of all ages. The major water-soluble protein has a molecular weight of 40,000 daltons (by HPLC), which is a dimer of 22,000 dalton chains (as seen by SDS-polyacrylamide electrophoresis). The unit peptide of fiber cell membranes (*i.e.*: water-insoluble, but SDS + DTT soluble) is also a 22,000 dalton chain. The squid lens contains low molecular weight (*i.e.*: <1000 daltons by pressure dialysis) chromophores with absorption maxima at 360 and 320 nm, and fluorescences that are excited at 350 and emit at 412 nm.

By using different lens structural and fiber cell membrane proteins compared to those of vertebrates, the squid has developed an alternative system to support both ocular transmission and accommodation.

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COPULATION BY HYPODERMIC INJECTION IN THE NUDIBRANCHS *PALIO ZOSTERAE* AND *P. DUBIA* (GASTROPODA, OPISTHOBRANCHIA)

BRIAN R. RIVEST

Department of Biological Sciences, State University of New York at Cortland, Cortland, New York 13045

ABSTRACT

The Pacific nudibranch *Palio* (= *Polycera*) *zosterae* and the Atlantic *P. dubia* copulate in a manner different from that of most nudibranchs. Nudibranchs are hermaphroditic and normally copulate reciprocally, with the intromittant organ of one inserted in the vaginal opening of the other. *P. zosterae* and *P. dubia* lack a complete vaginal duct. Copulation is usually reciprocal but involves the piercing of the body wall by a barbed, eversible penial cirrus. If the cirrus injects exogenous sperm into the body wall or the haemocoel, the sperm are phagocytosed by blood cells. If the cirrus penetrates the gonad, exogenous sperm may be injected into a spermatogenic acinus. After traveling down the hermaphroditic duct to the anterior reproductive organs, these sperm presumably are sorted from endogenous sperm and stored in the receptaculum seminis. The organization of the reproductive system in *Palio* differs markedly from that in members of the closely related genus *Polycera*.

INTRODUCTION

Nudibranchs are hermaphroditic gastropod molluscs with a complex reproductive system (Lloyd, 1952; Pruvot-Fol, 1960; Ghiselin, 1966; Hyman, 1967; Schmekel, 1971). The gonad lies in the posterior of the animal and is connected via a hermaphroditic duct to the anterior reproductive organs. These lie just inside the gonopore in the right anterior quandrant of the animal. In the anterior organs the exogenous and endogenous sperm are stored in separate structures, and prostatic secretions are produced and mixed with endogenous sperm during copulation. Eggs are fertilized there and surrounded by albumen, a capsule, and the mucous stroma of the egg mass during oviposition.

Even though nudibranchiate reproductive systems vary greatly in their organization (see review by Ghiselin, 1966), adults typically reciprocally copulate with their gonopores aligned so that the penis of one deposits sperm in the vagina of the other (Costello, 1938; Hyman, 1967). The exogenous sperm received during copulation may be initially stored in the bursa copulatrix, but is eventually stored in the receptaculum seminis for later use in fertilizing the animal's eggs (Thompson, 1961; Ghiselin, 1966). At all times the sperm are confined within the reproductive system of the donor or the recipient.

This paper presents observations on a novel mode of copulation in the phanerobranch nudibranchs *Palio* (= *Polycera*) *zosterae* (O'Donoghue, 1924) and *P. dubia* (Sars, 1824). These limaciform animals lack a complete vagina and copulate by piercing the body wall with a barbed penial cirrus. The fate of the injected sperm depends on where they are deposited within the recipient's body.

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Materials and Methods

Palio zosterae were collected during summer months in the shallow subtidal zone in Mitchell Bay, San Juan Island, Washington. The nudibranchs typically were found feeding on the bryozoan *Membranipora membranacea* (Linnaeus, 1767) which grows on the kelp *Laminaria* sp. Nudibranchs were maintained in sea tables at the Friday Harbor Laboratories, Friday Harbor, Washington, where their copulatory behavior was observed.

Specimens for histological examination were relaxed with isotonic $MgCl_2$, fixed in Bouin's, Hollande-Bouin's, or Zenker's fixative, embedded in paraffin, and serially sectioned at four to eight microns. Mounted deparaffinized sections were stained with either haematoxylin and eosin, Masson's trichrome, or Mallory-Heidenhain azon. To determine the fate of injected sperm, fifteen specimens were fixed without $MgCl_2$ treatment either during copulation, within one hour, or after 4, 8, 12, or 24 hours. These specimens were then serially sectioned and stained as described above.

Ten immature specimens were selected from collected nudibranchs. They were deemed immature due to their small size relative to adults ($\leq$3 mm *versus* $\geq$7 mm), the lack of externally visible ovotestes, and their lack of copulatory and egg laying behavior. Eight of these were isolated in dishes of sea water and fed *Membranipora*. The remaining two were maintained in one dish as controls. In four weeks all ten were considered sexually mature because of their adult size, externally visible ovotestes, and the copulatory and egg-laying behavior of the control animals. Two of the isolated nudibranchs were fixed without having been allowed to copulate and then prepared for serial sectioning. The others were paired and their copulatory behavior observed. Six were then fixed at various times up to 24 hours after copulation, embedded in paraffin and serially sectioned. Two were kept together for an additional two days to see if they laid eggs.

For comparative purposes, *Palio dubia* and three species from the closely related genus *Polycera* were examined. *Palio dubia* were collected subtidally on pier pilings where they were feeding on *Bowerbankia gracilis* Leidy, 1855, at Gerrish Island, Kittery, Maine, and in the nearby rocky intertidal zone. These nudibranchs were maintained at the University of New Hampshire, Durham, New Hampshire, where their copulatory behavior was observed. Four specimens were fixed and serially sectioned while others were dissected. Preserved specimens of the Pacific *Polycera tricolor* Robilliard, 1971, and the European *P. faeroensis* Lemche, 1929, and *P. quadralineata* (Müller, 1776) were obtained for dissection and histological examination. Radulae and jaws of all species were examined for taxonomic purposes.

Results

Copulatory behavior

Copulation in *Palio zosterae* involves the penetration of the partner's body wall by a penial cirrus. Two nudibranchs become sexually aroused upon contacting each other, and usually align head-to-tail with right sides opposing. A transluscent preputium everts through the external gonopore located anteriorly on each animals' right side (Figs. 1, 2). The vas deferens runs from the animal's interior to the most distal point of the preputium. The cirrus is housed within the vas deferens. Just posterior to the vas deferens is a vaginal inpocketing that ends blindly within the expanded preputium.

The distal end of the preputium is normally pressed against the side of the partner before the cirrus is everted. As the cirrus everts, proximally directed, serially arranged barbs covering the external surface of the cirrus are exposed (Fig. 3). The barbs appear

FIGURE 1. Two specimens of *Palio zosterae* reciprocally copulating by hypodermic injection, with the penial cirrus of each penetrating the body wall below the gills (g) of the other. Within the expanded preputium of the upper animal the vas deferens (vd) and the blind vaginal inpocketing (vi) can be seen. Each nudibranch is about 8–10 mm in length.

FIGURE 2. Non-reciprocal copulation in *Palio zosterae*. Moments earlier these two nudibranchs had been copulating reciprocally. The penial cirrus has partially everted from the tip of the preputium of the upper nudibranch. Each animal is about 8–10 mm in length.

to serve at least three functions. First, when the preputium is pressed against the partner's body wall and the cirrus begins to evert, the barbs roll in a radiating fashion over the lip of the elongating cirrus, tearing a hole in the body wall. Second, the barbs then anchor the proximal region of the cirrus in the epidermal tissue as the cirrus continues to evert. Third, the barbs hold the penis in place even if the partner starts to move away. When this happens, the nudibranch may be dragged along by its cirrus. During bouts of copulatory activity, the cirrus may be everted when the preputium is not pressed against the partner (Fig. 2). Under these circumstances the cirrus has been measured to evert up to one mm beyond the tip of the preputium in an eight mm long *P. zosterae*.

The site where the cirrus penetrates the partner's body wall varies, but since the majority of copulations are reciprocal, the point of penetration is usually in the posterior right flank of each nudibranch (Fig. 1). A cirrus piercing the body there is likely to penetrate the gonad, which lies a short distance beneath the body wall (Fig. 4). Hundreds of copulations have been observed, and never has the cirrus penetrated the vaginal inpocketing, the preputium, or a site within the external genital opening. Of 88 copulations where the point of penetration was recorded, 53% of the penetrations occurred in the posterior right flank, 26% in the posterior left flank, 7% in the head, 3% in the foot, and 11% in other locations such as the rhinophores, dorsal surface, and gills. Reciprocal copulations last one to six minutes, with the cirrus often being

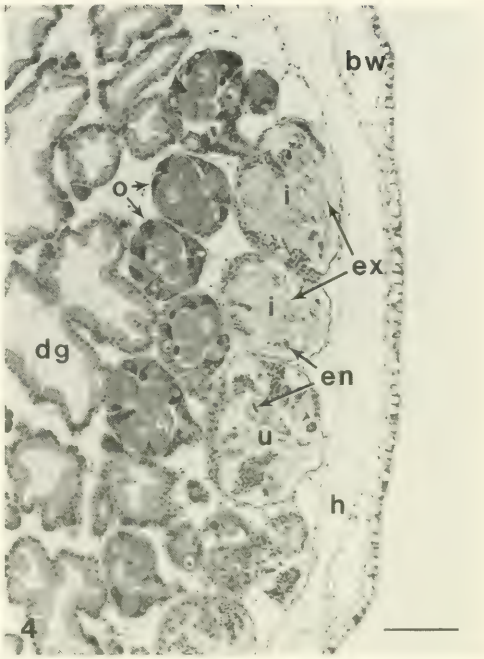

FIGURE 3. Scanning electron micrograph of a partially everted, barbed penial cirrus protruding from the tip of the ciliated preputium in *Palio zosterae*. Bar = 100 μm.

FIGURE 4. Paraffin section showing the organization of the visceral mass in *Palio zosterae*. The relative position of the male and female acini is opposite that found in *Polycera*. This animal was fixed shortly after copulation, and the upper two spermatogenic acini visible here are filled with exogenous sperm. bw—body wall, dg—digestive gland, en—endogenous sperm or spermatids, ex—exogenous sperm, h—haemocoel, i—injected spermatogenic acinus, o—oogenic acini, u—uninjected spermatogenic acinus. Bar = 100 μm.

retracted and reinserted at a new location several times during a copulatory bout. The cirrus is invaginated, disengaging its barbs, as it is withdrawn.

During copulation, peristaltic action of the muscular vas deferens moves sperm distally through the preputium into the cirrus. In some of the observed copulations, masses of sperm were deposited within the body wall and haemocoel of the partner. The sperm appeared as a white mass of various sizes up to 0.75 mm in diameter but usually much smaller. Some masses dissipated within 5–10 minutes; others remained visible through the body wall for up to six hours. Occasionally some sperm leaked out of the penetration wound or the tip of the cirrus as the penis was withdrawn from the partner.

Specimens of *Palio zosterae* kept in a dish appeared to be attracted to copulating conspecifics and stimulated to participate in copulatory activity. Thus a pair of nudibranchs engaging in copulation were soon joined by one to several others, with individuals in the center of the group being injected simultaneously by up to four others. Groups of three copulating *P. zosterae* were observed four times in the field, with the middle individual being injected in both the right and left sides.

The mature Atlantic *Palio dubia* is much longer than *P. zosterae* (13–25 mm *versus* 7–11 mm). Although fewer observations were made on *P. dubia*, copulation appears no different than in *P. zosterae*. During observed copulations in *P. dubia*, all

sides of the body were penetrated by the partner's cirrus, but the penetration site was usually in the posterior right flank during reciprocal copulation. Never was the cirrus inserted in the vaginal inpocketing or external gonopore.

Reproductive system anatomy in Palio

The reproductive systems of *Palio zosterae* and *P. dubia* differ only in the relative sizes of some of the reproductive structures. Therefore, the following anatomical description of the reproductive system applies to both species.

The internal organs of *Palio* consist of three large masses: the buccal mass containing the jaws and radula at the anterior end of the animal, the anterior (or distal) reproductive organs just inside the genital pore, and the posterior visceral mass beneath the gills. These masses fill much of the large body cavity, but large haemocoelic spaces remain between them. The reproductive system of *Palio* is illustrated in Figure 5.

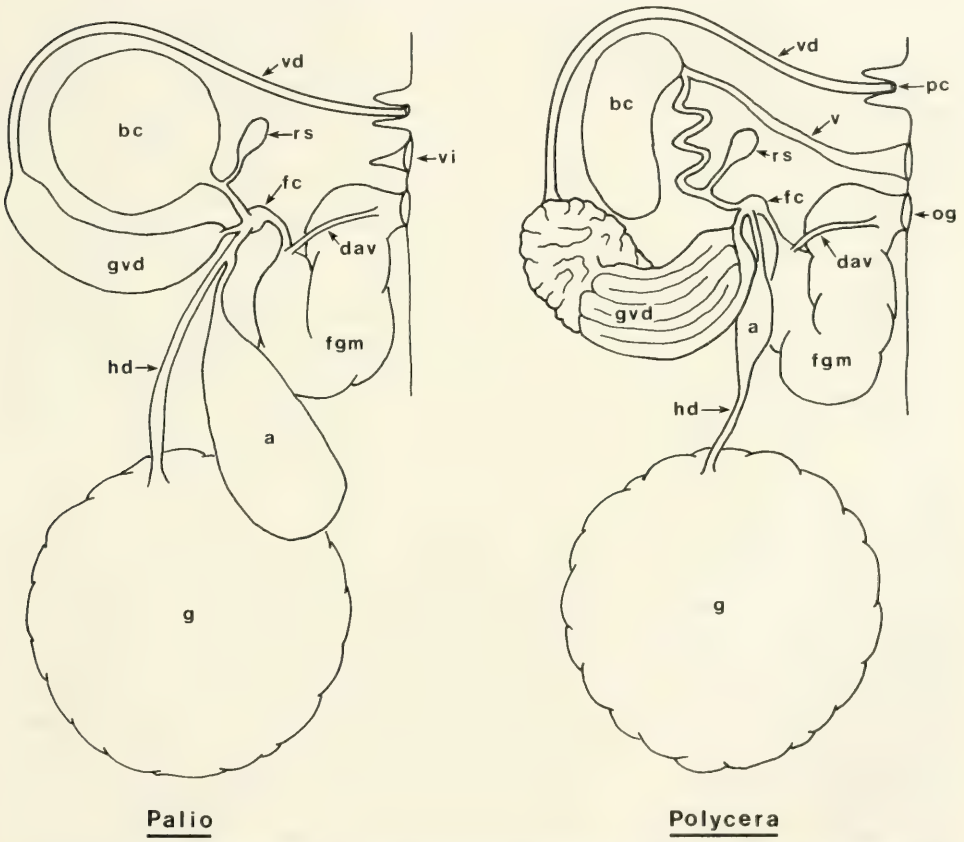

FIGURE 5. Schematic diagram of the reproductive systems of *Palio* and *Polycera,* based on observations on *Palio zosterae, P. dubia, Polycera quadralineata, P. faeroensis,* and *P. tricolor.* Dorsal view. The reproductive openings would normally be housed within an external common gonopore. a—ampulla, bc—bursa copulatrix, d—ductus-albumino-vestibularis, fc—fertilization chamber, fgm—female gland mass, g—visceral mass containing the gonad, gvd—glandular vas deferens, hd—hermaphroditic duct, og—oviducal gonopore, pc—partially everted penial cirrus, rs—receptaculum seminis, v—vaginal duct, vd—muscular vas deferens, vi—vaginal inpocketing.

The spherical visceral mass consists of a central stomach and digestive gland covered by the ovotestis. The ovotestis is organized into oogenic and spermatogenic acini. Several oogenic acini are connected via short necks to each spermatogenic acinus. Whereas there is little unoccupied space in the center of the oogenic acini due to the several developing ova bulging inward, the spermatogenic acini have a large lumen occupied peripherally by spermatogonia and spermatocytes with some maturing spermatids suspended towards the center. The relative position of these male and female acini in *Palio* is opposite that found in *Tritonia hombergi* (Thompson, 1961) and *Polycera* (pers. obs.), with the spermatogenic acini lying peripherally, instead of centrally. These spermatogenic acini lie just underneath the tissue covering the visceral mass. Beneath the spermatogenic acini are most of the oogenic acini (Fig. 4). A few oogenic acini are found at the surface of the visceral mass, but not on the right lateral surface where penetration by a cirrus is most likely to occur. A branch of the hermaphroditic duct arises from each spermatogenic acinus. This branch joins others to form a common hermaphroditic duct that leaves the anterior margin of the visceral mass and descends into the center of the anterior reproductive organs. Because of the gonadal arrangement, ova released from oogenic acini must pass through the spermatogenic acinus before reaching the hermaphroditic duct.

Near the middle of the anterior reproductive organ mass, the hermaphroditic duct gives off a short duct that reflects posteriorly and enlarges into a lacrimate ampulla (Fig. 4) in which endogenous sperm are presumably stored (Beeman, 1970a, b; Ghiselin, 1966). The ampulla measures about 1.5 mm in diameter and 3.5 mm long in a 10 mm long *Palio zosterae*. The white sperm mass in such a large ampulla is visible through the body wall. The ampulla was never empty, but was swollen with sperm even after an animal had copulated repeatedly.

Shortly after the ampullar duct is given off, the hermaphroditic duct splits into the vas deferens and the proximal oviduct. The vas deferens becomes enlarged, glandular, and somewhat flattened as it wraps around the posterior and medial sides of the bursa copulatrix. The columnar, ciliated prostatic gland cells of this region produce eosinophilic secretions that can be seen in the lumen of the duct in sections of animals that were sexually active when fixed. This prostatic region of the vas deferens attenuates into a muscular duct which may coil several times before it reaches the external gonopore. When not in use, the barbed cirrus is housed inverted within the distal region of the vas deferens.

Beyond the bifurcation of the hermaphroditic duct into the vas deferens and proximal oviduct, the proximal oviduct immediately enlarges into a fertilization chamber, the interior walls of which are ciliated and thrown into numerous folds. In some specimens the proximal oviduct is essentially non-existent, with the vas deferens splitting off from the fertilization chamber near the point where the hermaphroditic duct enters. Two other ducts join the fertilization chamber. One leads medially to the bursa copulatrix and receptaculum seminis. The second duct, the distal oviduct, reflects posteriorly along the descending hermaphroditic duct and expands into the albumen gland which leads to the capsule gland of the female gland mass. This then turns anteriorly into the large mucous gland leading to the oviducal gonopore. A short, tiny (25 μm in diameter), ciliated duct bypasses the female gland mass by connecting the proximal end of the female gland mass directly to the distal end. This is the ductus-albumino-vestibularis first described in *Polycera quadralineata* by Pohl (1905).

The spherical bursa copulatrix, presumably the initial storage site for exogenous sperm (Ghiselin, 1966) or possibly a gametolytic organ (Thompson and Bebbington, 1969; Beeman, 1970b), occupies much of the medial portion of the anterior repro-

ductive organ mass. It may be 0.7 mm in diameter in a *P. zosterae* 7 mm long. The epithelial cells of the bursa copulatrix appear cuboidal and slightly glandular, and are ciliated near the opening to the duct. In virgin or recently mated animals, the bursa copulatrix contained a few sperm and some unidentifiable material. A few hours after copulations where sperm were injected into the gonad, the bursa copulatrix was packed with sperm.

A lacrimate receptaculum seminis, a storage organ for exogenous sperm (Beeman, 1970b), is at the end of a short duct that joins the distal end of the duct to the bursa copulatrix. In contrast to the ampulla and bursa copulatrix, the receptaculum seminis is a small structure only about 0.15 mm long and 0.06 mm in diameter in *P. zosterae*. It may contain loosely or densely packed sperm, the heads of which are not embedded in or aligned with the sparsely ciliated epithelial lining as they are in other opisthobranch species (*e.g.*, Ghiselin, 1966; Thompson, 1966; Beeman, 1970a, b).

The vaginal inpocketing visible in the expanded preputium of living animals can be followed in the serial sections. This inpocketing of non-glandular cells ends blindly, with no trace of a connection or vestigial structure continuing internally. Also, no duct or remnant of the duct connecting the bursa copulatrix or receptaculum seminis or their ducts to the gonopore exists. Only the vas deferens and the distal oviduct connect the reproductive organs to the outside.

Fate of injected sperm

Observations of sperm being injected into the haemocoel stimulated an initial hypothesis that exogenous sperm enter the reproductive system of the recipient through an opening, such as a ciliated coelomostome, which collected the sperm from the haemocoel and moved them into an appropriate duct of the reproductive system. Three observations argue against this hypothesis. First, no such opening was seen in sectioned material, although the possibility of a transient opening exists. Second, blood cells aggregate around clusters of sperm in the haemocoel, interstices of the ovotestis or in the body wall (Fig. 6). These blood cells appeared to be phagocytosing the wayward sperm; sperm heads were seen within some blood cells. Third, numerous examples of acini injected with exogenous sperm were found.

In order for a cirrus to inject sperm into a partner's ovotestis, it must penetrate the body wall, traverse part of the haemocoel, penetrate the tissue covering of the visceral mass, and puncture an acinus. This represents a minimum distance of 100 μm in a 7 mm long *P. zosterae*. In serial sections of animals fixed shortly after copulation, wounds in the body wall and tissue covering the visceral mass were found next to punctured spermatogenic acini filled with sperm (Fig. 7). In some animals, sperm were found near the path of penetration in the body wall, haemocoel, and spaces around the ovotestis. Injected spermatogenic acini can be distinguished from non-injected ones by the number and organization of the spermatozoa they contain. Uninjected acini have no or few unattached spermatozoa, but have maturing spermatids suspended in arrays in the central lumen. Much empty space surrounds these arrays. In contrast, injected acini are packed with loose spermatozoa (Figs. 5, 7). Some copulations result in such volumes of sperm being injected into a spermatogenic acinus that some sperm are forced through the hermaphroditic duct into adjacent male acini. Sperm were also found in the common hermaphroditic duct and in the fertilization chamber in some animals with a recently injected gonad.

Prostatic secretions are injected along with sperm during copulation in *Palio*. In serially sectioned specimens fixed within eight hours of copulation, the eosinophilic prostatic secretions were found in the body wall, haemocoel, interstices of the visceral

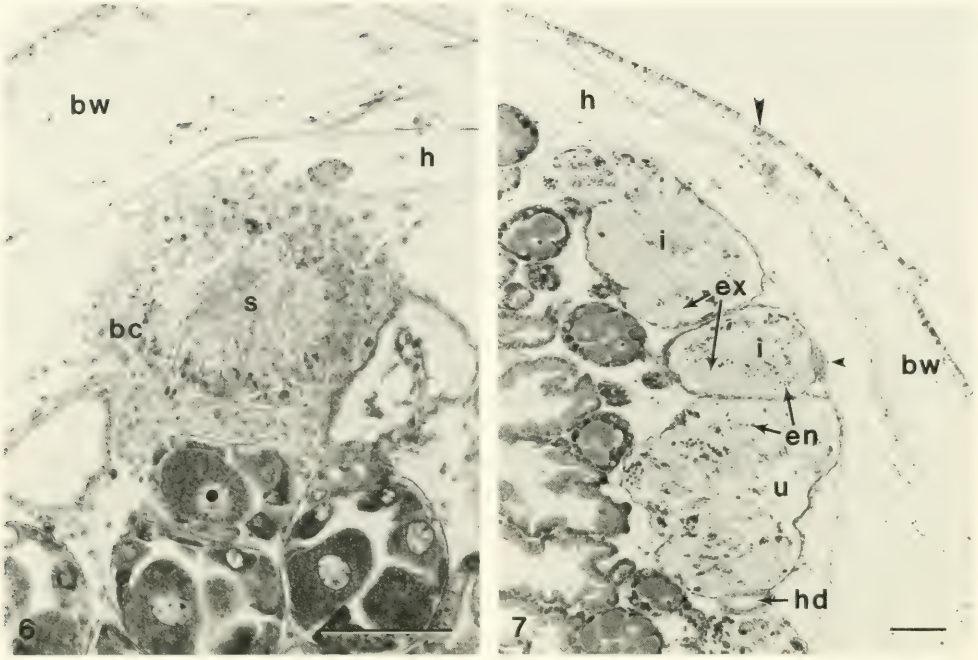

FIGURE 6. Sperm not injected into the gonad in *Palio* are phagocytosed by blood cells. This paraffin section of *P. zosterae* shows a mass of sperm that was injected into a peripheral interstice of the ovotestis and is now surrounded by blood cells. bc—blood cells, bw—body wall, h—haemocoel, s—sperm. Bar = 100 μm.

FIGURE 7. Paraffin section through the visceral mass of *Palio zosterae* showing the pathway by which a penial cirrus penetrated the body wall (large arrowhead) and gonad (small arrowhead). This section shows two lobes of the same injected spermatogenic acinus filled with unoriented exogenous sperm. The broken, outwardly displaced section of the epidermis is an artifact. bw—body wall, en—endogenous sperm or spermatids, ex—exogenous sperm, h—haemocoel, hd—branch of the hermaphroditic duct, i—injected spermatogenic acinus, u—uninjected spermatogenic acinus. Bar = 100 μm.

mass, and ovotestis. Sperm were mixed in with the prostatic secretions in animals that were fixed immediately after copulation. In other animals, injected secretions not within the gonad were without sperm, and were found concentrated in body spaces as if they had settled there due to gravity or due to the circulation of the blood. Prostatic secretions injected into the ovotestis appeared to be rapidly transported to the bursa copulatrix. Animals often had exogenous sperm still within spermatogenic acini and the hermaphroditic duct when the only hint of prostatic secretions within the reproductive system was eosinophilic globules within the bursa copulatrix.

The eight specimens of *P. zosterae* reared to maturity in isolation never laid egg masses while isolated. When placed together, they copulated by hypodermic injection as described above. Most of these animals were fixed shortly after copulation, but the two animals that were kept together for two days each laid a fertile egg mass. They had copulated repeatedly by hypodermic injection, but did not appear to copulate in a manner considered normal in other nudibranchs in which the penis enters a vaginal opening.

Serial sections of the two specimens of *Palio zosterae* reared in isolation but never allowed to copulate revealed in each an ampulla swollen with sperm, a bursa copulatrix

containing only a few sperm and unidentifiable debris, and an empty receptaculum seminis. In isolation animals that were fixed after being permitted to copulate, large numbers of unoriented sperm were seen in punctured spermatogenic acini. Sperm were found in the receptaculum seminis within four hours of copulation. In all of the mated isolation animals some of the injections hit the gonad while others missed.

Reproductive anatomy of Polycera *spp.*

To compare the reproductive systems of *Palio zosterae* and *P. dubia* with that of other polycerid nudibranchs, specimens of *Polycera quadralineata, P. tricolor,* and *P. faeroensis* were serially sectioned and dissected. Aspects of the reproductive anatomy of *P. quadralineata* have been described by Pohl (1905), of *P. tricolor* by Robilliard (1971), and of *P. faeroensis* by Odhner (1941). The reproductive system of these three species of *Polycera* is illustrated in Figure 5. It is fundamentally organized like that in *Palio* except for four major differences:

First, the oogenic acini are peripheral to the spermatogenic acini, like that previously described for *Tritonia hombergi* (Thompson, 1961).

Second, the ampulla is not a separate sac as in *Palio,* but is just an enlarged region of the hermaphroditic duct. Also, in the three species of *Polycera* that were serially sectioned and dissected, the ampulla was relatively much smaller than in *Palio.*

Third, the prostate is more complex in *Polycera* than in *Palio* where it is simply an enlarged glandular region of the proximal vas deferens. In *Polycera,* the prostate has two distinct glandular regions. In the first glandular region the vas deferens is folded back on itself several times. The second region occurs immediately after the first, but consists of an area of highly convoluted walls lined by cells with different staining characteristics than those of the first region.

Fourth, a complete vaginal duct connects the vaginal opening in the gonopore complex with the bursa copulatrix, joining the bursa at the site where the duct to the fertilization chamber enters. The vaginal duct lies posterior to the cirrus and anterior to the oviducal pore, a position analagous to that of the vaginal inpocketing in *Palio.*

Discussion

The taxonomic position of *Palio* (= *Polycera*) *zosterae* needs clarification. The genus *Palio* was separated from the genus *Polycera* by Gray (1857), with *Palio* possessing a tuberculate, as opposed to a digitate, frontal margin. Although Eliot (1910), Lemche (1929), and Odhner (1941) suggested that *Palio* should not remain separate from *Polycera* due to the variable nature of external differences, *Palio* has remained in use (*e.g.,* Swennen, 1961; Thompson and Brown, 1976). O'Donoghue described the Pacific nudibranch I have investigated as *Polycera zosterae* in 1924, but based on its external morphology it belongs in Gray's genus *Palio.* The evidence provided above suggests that differences in the reproductive system can also be used to separate members of the genus *Palio* from *Polycera: Palio* lacks a vaginal duct, possesses an ampullar sac, and has spermatogenic acini peripherally located in the ovotestis. In this context, an examination of the reproductive systems of *Palio pallida* Bergh, 1880, *P. parvula* Burn, 1958, and *P. amakusana* Baba, 1960, would be useful.

Copulation by hypodermic injection in *Palio zosterae* and *P. dubia* contrasts strikingly to the reciprocal intromission of a penis into a vaginal duct found in other nudibranchs (Costello, 1938; Haefelfinger, 1960; Rutowski, 1983). In *Palio,* the cirrus

penetrates the body wall to inject sperm either into the gonad, or into the haemocoel where the sperm are phagocytosed. The phagocytosed sperm may provide some nourishment, albeit small, to the recipient. In some non-molluscan species, material donated during mating contributes to the energy and material needs of the recipient (Hinton, 1964; Boggs and Gilbert, 1979).

Whereas copulation by hypodermic injection has not been previously reported in nudibranchs, it has for some ascoglossan opisthobranchs. The ascoglossans *Limapontia cocksi* and *L. capitata* (Gascoigne, 1956) and *Stiliger fuscatus* (Gascoigne, 1978) have a pseudodiaulic reproductive system. No vaginal opening exists but reciprocal copulation occurs with a penial stylet piercing the body wall and depositing sperm in the underlying bursa copulatrix. Alignment of the copulating animals in these species is essential for the bursa to be pierced. In two other ascoglossan species, *Elysia maoria* (Reid, 1964) and *Alderia modesta* (Hand and Steinberg, 1955; pers. obs.), copulation is also by hypodermic injection, but there is no preferred point of injection where an internal organ would be consistently pierced. The fate of injected sperm in these species is unknown.

Assuming that exogenous sperm fertilize eggs, the mode of copulation in *Palio* has a risk that sperm will not be injected into the partner's gonad and hence be wasted. However, this risk is minimized by the animals' behavior. Reciprocal copulation in *Palio* is not requisite but is the norm. During reciprocal copulation the cirrus penetrates the right flank where it is likely to hit the ovotestis.

The selective advantages of the mode of copulation found in *Palio* is enigmatic. *Palio* has lost the complete vaginal duct found in other nudibranchs. Regression in structure and function in the reproductive system is found in some interstitial molluscs, presumably because of space limitations (Swedmark, 1968). Such a space constraint is unlikely in *Palio zosterae,* which is larger than many other species of normally copulating nudibranchs. From an energetic standpoint, the mode of copulation exhibited by *Palio* may be more expensive than the normal mode because of misaimed injections. *Palio* has a relatively large ampulla compared with that in the normally copulating *Polycera,* suggesting that more sperm are needed per successful copulation.

Exogenous sperm are injected into the spermatogenic acini of *Palio* where endogenous sperm are made. With the techniques utilized in this study, endogenous sperm could not be distinguished from exogenous sperm in places other than recently injected acini. Therefore, what happens to sperm injected into the gonad can only be hypothesized based on the observations of individuals reared in isolation along with assumptions of functional anatomy of the reproductive system based on information from the literature (*e.g.,* Ghiselin, 1966; Beeman, 1970a, b). Both endogenous and exogenous sperm travel down the hermaphroditic duct to the anterior reproductive organs. Endogenous sperm are passed into the ampulla while exogenous sperm travel through the fertilization chamber to the receptaculum seminis. The reproductive system in *Palio* thus may have endogenous and exogenous sperm coexisting in the gonad and hermaphroditic duct. How the reproductive system distinguishes exogenous from endogenous sperm in its sorting process is unknown. One possible difference between the two types of sperm is that the exogenous sperm may have been capacitated by secretions from the donor's prostate (Thompson, 1966). But if sperm capacitation due to prostatic secretions results in character differences used for sperm sorting, and if the action of prostatic secretions is not self-specific, then a problem arises. Since prostatic secretions are injected along with sperm during copulation, endogenous sperm might be capacitated, stored in the receptaculum seminis, and used for fertilizing eggs, resulting in self-fertilization. However, the chances of fertilization by endogenous sperm capacitated by exogenous prostatic secretions is low because few mature en-

dogenous sperm are in the spermatogenic acini or hermaphroditic duct at any given time. Therefore, few mature endogenous sperm would be exposed to injected prostatic secretions.

The movement of *Palio zosterae* to nearby copulating conspecifics and their subsequent involvement in the copulatory activity suggest a chemically mediated attraction. Chemical signals apparently stimulate aggregation and breeding activity in *Aplysia*, an anaspidian opisthobranch which mates in long chains (Audesirk, 1977; W. Aspey, pers. commun.). Nudibranchs normally mate in pairs and not in chains (Costello, 1938; Hyman, 1967), but *Palio* is unlike other nudibranchs. The mode of copulation in *Palio* permits more than two individuals to participate simultaneously. By attracting others, mating individuals may increase the chance that they will be successfully inseminated.

Presumably *Palio* evolved from a polycerid ancestor possessing a triaulic reproductive system. The layout of the reproductive system in *Palio* is like that in *Polycera* except for the four differences mentioned above. The vaginal inpocketing in *Palio* has no apparent function, but is in the same relative position as the vaginal opening in *Polycera*. The inpocketing is probably a remnant of an ancestral vaginal opening. The loss of the vaginal duct might have been the first alteration in the ancestral triaulic reproductive system, and other modifications arose as a consequence of the new mode of copulation. The barbed cirrus used for penetrating the body wall during copulation in *Palio* is also a characteristic of members of the genus *Polycera* (Bergh, 1880; Odhner, 1941; Pruvot-Fol, 1954; Marcus and Marcus, 1967; Robilliard, 1971). An armed, evaginating cirrus is probably ancestral in *Palio*, and was therefore preadapted for use as a penetrating organ during copulation by hypodermic injection.

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LITERATURE CITED

AUDESIRK, T. E. 1977. Chemoreception in *Aplysia californica*. III. Evidence for pheromone influencing reproductive behavior. *Behav. Biol.* **20:** 235–243.

BEEMAN, R. D. 1970a. The anatomy and functional morphology of the reproductive system in the opisthobranch mollusk *Phyllaplysia taylori* Dall, 1900. *Veliger* **13:** 1–31.

BEEMAN, R. D. 1970b. An autoradiographic study of sperm exchange and storage in a sea hare, *Phyllaplysia taylori*, a hermphaphroditic gastropod (Opisthobranchia: Anaspidia). *J. Exp. Zool.* **175:** 125–132.

BERGH, R. 1880. On the nudibranchiate gasteropod Mollusca of the North Pacific Ocean, with special reference to those of Alaska. *Proc. Acad. Natl. Sci. Phila.* pt. **2:** 40–127.

BOGGS, C., AND L. E. GILBERT. 1979. Male contribution to egg production in butterflies: evidence for transfer of nutrients at mating. *Science* **206:** 83–84.

COSTELLO, D. P. 1938. Notes on the breeding habits of the nudibranchs of Monterey Bay and vicinity. *J. Morphol.* **63:** 319–344.

ELIOT, C. N. E. 1910. *A Monograph of the British Nudibranch Mollusca, Part 8 (Supplement)*. Ray Society, London. 198 pp.

GASCOIGNE, T. 1956. Feeding and reproduction in the Limapontiidae. *Trans. R. Soc. Edinb.* **63:** 129–151.

GASCOIGNE, T. 1978. The internal anatomy of *Stiliger fuscatus* (A. Gould, 1870) (Opisthobranchia: Sacoglossa). *Zool. J. Linn. Soc.* **63:** 265–274.

GHISELIN, M. T. 1966. Reproductive function and the phylogeny of opisthobranch gastropods. *Malacologia* **3:** 327–378.

GRAY, J. E. 1857. *Guide to the Systematic Distribution of the Mollusca in the British Museum. Part I.* Taylor and Francis, London. 230 pp.

HAEFELFINGER, H. 1960. Beobachtungen an *Polycera quadralineata* (Müller) (Mol. Nudibr.). *Rev. Suisse Zool.* **67:** 101–117.

HAND, C., AND J. STEINBERG. 1955. On the occurrence of the nudibranch *Alderia modesta* (Loven, 1844) on the central California Coast. *Nautilus* **69:** 22–28.

HINTON, H. E. 1964. Sperm transfer in insects and the evolution of haemocoelic insemination. Pp. 45–107 in *Insect Reproduction,* K. C. Highman, ed. Symp. R. Entomol. Soc. Lond.

HYMAN, L. H. 1967. *The Invertebrates, Vol. VI: Mollusca I.* McGraw-Hill Book Company, Inc., New York. 792 pp.

LEMCHE, H. 1929. Gastropoda opisthobranchiata. *Zoology Faroes* **53:** 1–35.

LLOYD, H. M. 1952. A study of the reproductive systems of some opisthobranch molluscs. Doctoral dissertation, University of London.

MARCUS, E., AND E. MARCUS. 1967. American opisthobranch molluscs. Part II. Opisthobranchs from the Gulf of California. *Stud. Trop. Oceanogr. (Miami)* **6:** 141–256.

ODHNER, N. H. 1941. New polycerid nudibranchiate Mollusca and remarks on this family. *Goteb. K. Vetensk. Vitter Hetssamh. Handl. Sjatte Foljden Ser. B* **1:** 1–20.

O'DONOGHUE, C. H. 1924. Notes on the nudibranchiate mollusca from the Vancouver Island region. IV. Additional species and records. *Trans. R. Can. Inst.* **15:** 1–33.

POHL, H. 1905. Über den feinern Bau des Genitalsystems von *Polycera quadralineata. Zool. Jahrb. Abt. Anat. Ont. Tiere* **21:** 427–452.

PRUVOT-FOL, A. 1960. Les organes génitaux des opisthobranches. *Arch. Zool. Exp. Gén.* **99:** 135–224.

REID, J. D. 1964. The reproduction of the sacoglossan opisthobranch *Elysia maoria. Proc. Zool. Soc. Lond.* **143:** 365–393.

ROBILLIARD, G. A. 1971. A new species of *Polycera* (Opisthobranchia: Mollusca) from the northeastern Pacific, with notes on other species. *Syesis* **4:** 235–243.

RUTOWSKI, R. L. 1983. Mating and egg mass production in the aeolid nudibranch *Hermissenda crassicornis* (Gastropoda: Opisthobranchia). *Biol. Bull.* **165:** 275–285.

SCHMEKEL, L. 1971. Histologie und Feinstruktur der Genitalorgane von Nudibranchiern (Gastropoda, Euthynura). *Z. Morphol. Tiere* **69:** 115–183.

SWEDMARK, B. 1968. The biology of interstitial Mollusca. *Symp. Zool. Soc. Lond.* **22:** 135–149.

SWENNEN, C. 1961. Data on distribution, reproduction and ecology of the nudibranchiate molluscs occurring in the Netherlands. *Neth. J. Sea Res.* **1,1/2:** 191–240.

THOMPSON, T. E. 1961. The structure and mode of functioning of the reproductive organs of *Tritonia hombergi* (Gastropoda Opisthobranchia). *Q. J. Microsc. Sci.* **102:** 1–14.

THOMPSON, T. E. 1966. Studies on the reproduction of *Archidoris pseudoargus* (Rapp) (Gastropoda, Opisthobranchia). *Phil. Trans. R. Soc. Lond. B Sci.* **250:** 343–375.

THOMPSON, T. E., AND A. BEBBINGTON. 1969. Structure and function of the reproductive organs of three species of *Aplysia* (Gastropoda: Opisthobranchia). *Malacologia* **7:** 347–380.

THOMPSON, T. E., AND G. H. BROWN. 1976. *Synopsis of the British fauna (New Series). No. 8: British Opisthobranch Molluscs.* Academic Press, London. 203 pp.

Reference: *Biol. Bull.* **167**: 555–567. (December, 1984)

CRITICAL WEIGHTS FOR METAMORPHOSIS IN THE TOBACCO HORNWORM, *MANDUCA SEXTA*

LOUIS SAFRANEK AND CARROLL M. WILLIAMS

The Biological Laboratories, Harvard University, Cambridge, Massachusetts 02138

Abstract

Fifth-instar larvae of the tobacco hornworm underwent a supernumerary larval molt under several feeding regimens. Supernumerary molting occurred only when the molt was initiated at a live weight less than about 3 g; at higher weights pupation took place. The supernumerary sixth instars were normal in appearance and behavior and went on to form normal pupae only when the fifth instar had begun at weights less than 1 g. Otherwise the supernumerary sixth instars exhibited precocious metamorphosis of their crochets and imaginal discs and failed to undergo normal pupation. Thus, the commitment to pupal development appears to occur in stages. A first stage, initiated at a weight of about 1 g, commits the crochets and imaginal discs to metamorphosis at the next molt. A second stage, entered at a weight of about 3 g, permits the full and complete pupation of the remaining tissues; this transition probably reflects the elimination of juvenile hormone (JH). Starvation apparently alters the normal patterns of ecdysone production, breakdown, or sensitivity so as to elicit ecdysone-dependent development at lower than normal weights. When starvation results in fifth-instar larvae initiating a developmental response at weights below 3 g, a supernumerary larval molt occurs. But in this case the critical effect of starvation is on the timing of the molt rather than on the JH titer, since the latter even in normally fed early fifth instars is high at weights below 3 g.

Introduction

Metamorphosis of the tobacco hornworm *Manduca sexta* normally occurs at the conclusion of the fifth instar. Nevertheless, experimental regimens of malnutrition can elicit one or even two additional larval molts (Nijhout, 1975a; Jones *et al.*, 1980; Cymborowski *et al.*, 1982). These supernumerary molts, like the four normal ones, require the concerted action of ecdysone and juvenile hormone (JH). Previous studies of these molts have focused primarily on the regulation of the hemolymph JH titer and the activity of the corpora allata (CA), both of which were high in fifth-instar larvae undergoing an extra larval molt but low in individuals initiating metamorphosis (Nijhout and Williams, 1974b; Nijout, 1975b; Bhaskaran and Jones, 1980; Cymborowski *et al.*, 1982). The high JH titer has been advanced as both a necessary and a sufficient cause of the extra larval molts (Bhaskaran and Jones, 1980; Cymborowski *et al.*, 1982).

The present study addresses this phenomenon anew. We have examined the effects of certain feeding regimens on the development of final-instar hornworms. The outcomes of these experiments argue against the earlier conclusion that supernumerary molting of malnourished fifth-instar larvae results from an atypical elevation of the JH titer. In the accompanying paper (Safranek and Williams, 1984) we demonstrate

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Abbreviations: JH, juvenile hormone; CA, corpora allata; LD, long day; SD, short day.

that larvae are regularly able to molt at much lower than predicted weights when they are malnourished. Here we suggest that a similar downward adjustment occurs as a response to malnourishment in larvae starved in the nominally final instar. When the adjustment is of sufficient magnitude, the molt begins at low body weights where the JH titer is high even in normally fed larvae. The resulting supernumerary larval molt thus reflects not an unusual variation in the JH titer but rather an alteration in the endocrine axis surrounding ecdysone that leads to molting at body weights below a threshold critical for metamorphosis.

Materials and Methods

Hornworms were reared at 25°C on an artificial diet as described by Truman (1972) and Bell and Joachim (1976) under either short-day (SD, 12L:12D) or long-day (LD, 17L:7D) photoperiods. Under these conditions the larvae routinely underwent five larval instars. Time-of-day was arbitrarily referenced to lights-off at midnight (24:00 = 00:00). The timing of events in the life cycle was as described by Truman (1972) and Truman and Riddiford (1974) except that we have termed the first 24 h of each instar Day 1 rather than Day 0.

Several different feeding regimens were employed in these experiments. In the "steady-weight regimen" larvae were weighed and returned to individual, clean, capped plastic containers which contained no food. Thereafter at daily intervals each individual was weighed and provided with an amount of diet sufficient to restore it to near its original weight. This regimen was maintained until molting or metamorphosis was initiated, as signaled, respectively, by apolysis of the head capsule or exposure of the dorsal vessel. In the "starvation-refeeding regimen" early fifth-instar larvae were weighed and returned to clean, individual, capped plastic containers where they were starved for one or more days. Thereafter they were restored to normal diet *ad lib* and observed daily for the onset of molting or metamorphosis.

Individuals molting to the sixth instar were subsequently observed under the dissecting microscope for a number of characters: the presence of pupal cuticle about the ocelli, antennae, and mouthparts; the appearance of pupal cuticle on the imaginal wing discs as seen through the translucent body wall; and the number, size, and shape of crochets on the prolegs. In the case of larvae dying prior to the onset of metamorphosis, the wing discs were excised to ascertain the presence or absence of a tanned, brittle, rugose, pupal-type cuticle.

Results

Effects of a "steady-weight" feeding regimen on final instar larvae

LD fifth-instar larvae were subjected to a steady-weight feeding regimen as described under Materials and Methods. Individuals were selected from each day of the instar up to 22:00 on the fourth day; all were therefore at least 4 h prior to the initiation of the pre-metamorphic endocrine events that culminate in the onset of the wandering period on the sixth day (Riddiford and Curtis, 1978). Larvae were maintained by daily feedings within .05 g of their weight at the outset of the regimen. All larvae underwent a molt. As depicted in Figure 1, those maintained at weights under 2.25 g formed supernumerary sixth-instar larvae after not less than 10 days (range: 10–25 days). By contrast, those held at steady weights ranging from 3.75 to 8.25 g exposed the dorsal vessel after less than 7 days and then formed normal pupae. Individuals held at weights between 2.75 and 3.25 g showed a variety of responses: some molted to sixth-instars, others wandered and subsequently formed normal albeit miniature pupae, still others molted to larval-pupal intermediates.

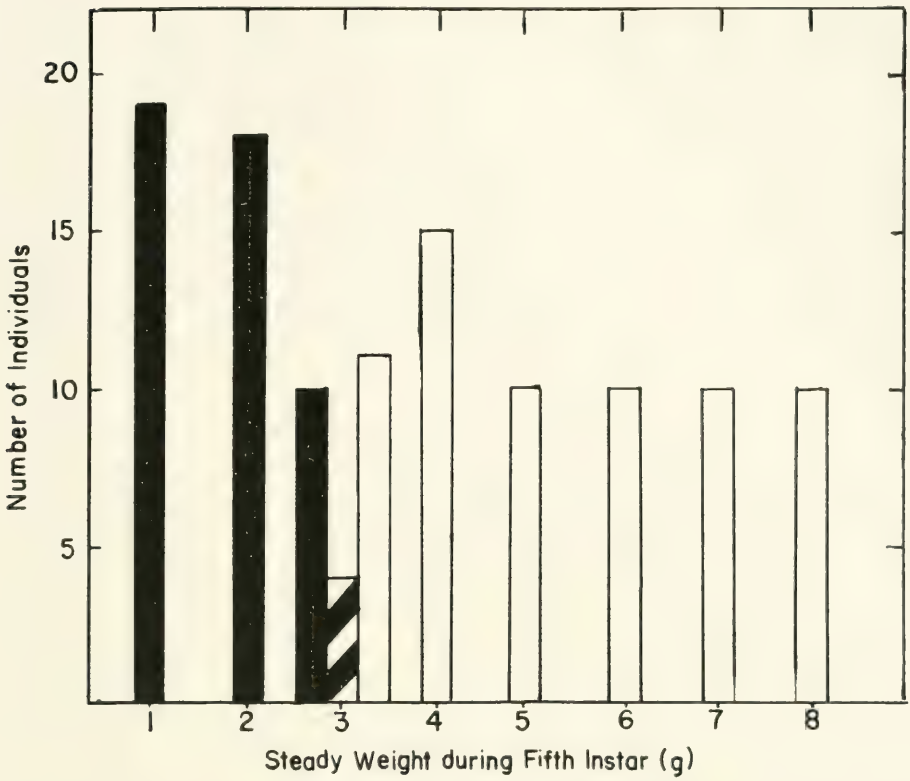

FIGURE 1. Transition from supernumerary larval molting to metamorphic development between 2 and 4 g in larvae maintained on a steady-weight regimen during the fifth instar. The number of larvae undergoing each type of development is shown as a function of the maintenance weight in the fifth instar. The various developmental outcomes were classified into 3 groups: (1) supernumerary sixth-instar larvae (black bars) lacked any traces of tanned pupal-like cuticle on epidermal structures other than the imaginal discs; (2) larval-pupal intermediates (hatched bars) exhibited regions of both larval-like and pupal-like cuticle; and (3) pupae (white bars) were smaller than normal but otherwise essentially normal in appearance and without traces of clear, flexible larval cuticle. Weight categories ranged ±0.25 g about the integral weights depicted in the figure. Details of this steady-weight regimen are described under Materials and Methods and Results.

Larvae molting to supernumerary sixth instars were never normal and none went on to pupate. The majority failed to undergo ecdysis or did so only partially and died without resuming feeding. The few which underwent successful ecdysis fed sporadically and ultimately died without further development. Attempts to salvage them by manually removing the old cuticle were to no avail since none resumed feeding. All exhibited one or more morphological abnormalities previously encountered in sixth instars resulting from juvenoid treatment of fifth instars (Trumen *et al.*, 1974). In the least affected individuals the crochets of one or more pairs of prolegs were noticeably reduced in size and number. The most abnormal lacked crochets entirely and had formed pupal cuticle on uneverted imaginal wing disks as well as on and about the ocelli and the everted imaginal discs of the antennae and mouthparts. Fewer abnormalities occurred when the maintenance weight was closer to 1 than to 3 g.

Among larvae destined to form larval-pupal intermediates an occasional individual exposed the dorsal vessel just prior to or even during apolysis and head capsule

slipping, but most did not. Regardless of the degree to which the dorsal vessel was exposed, all intermediates not only bore the just-described abnormalities characteristic of sixth-instar larvae but also displayed well-tanned, pock-marked pupal-like cuticle over variable but often extensive areas of the head, thorax, and abdomen.

In additional experiments we examined the development of about 100 fifth instars under short-day rather than the previous long-day conditions. Although the general pattern of response was the same as encountered in LD fifths, the critical weight for switching from a molting to a metamorphic response was nearer 2 than 3 g as under LD conditions. This difference withstood repeated scrutiny.

These experiments suggested that attainment of a threshold weight of about 2–3 g was a prerequisite for normal metamorphosis, but also that some metamorphic features were still to be found in the supernumerary larvae formed by fifth instars molting at even lower weights.

Effects of starvation-refeeding schemes on fifth-instar larvae

We next employed starvation-refeeding regimens of the type used to generate supernumerary instars in previous studies (Jones *et al.*, 1980; Cymborowski *et al.*, 1982). Thirty LD fifth-instar larvae were starved beginning on Day 1 at weights of 1.5–2.0 g. Thereafter groups of 10 were permitted to feed *ad lib* beginning on Days 4, 5, or 6 at about 18:00. All individuals were weighed daily at about 18:00 and observed for apolysis or dorsal vessel exposure.

The observations summarized in Figure 2 reveal that larvae destined to undergo a supernumerary larval molt typically grew more slowly than those that metamorphosed. The groups differed in two additional respects. Individuals undergoing a supernumerary molt were distinctly smaller at the time of apolysis (range: 2.5–5 g) than were metamorphosing larvae at the time of dorsal vessel exposure (range: 5–10 g). Moreover, apolysis occurred late on the fourth day of refeeding, whereas dorsal vessel exposure took place on the fifth or sixth day. If, in fact, initiation of the supernumerary molt in the refed larvae took place some 12–18 h prior to apolysis as is the case in normal larvae at the conclusion of the fourth instar (Truman, 1972; Cymborowski *et al.*, 1982), the molt would have been initiated typically at weights of about 3 to 3.5 g between Days 3 and 4 after feeding was resumed. The refed fifths that underwent metamorphosis were distinctly larger at a similar point after resumption of feeding, typically weighing 4–6 g between Days 3 and 4 of refeeding.

In a further series of experiments 30 LD fifth-instar larvae similar to those above were starved for 3, 4, or 5 days at the outset of the instar. But in this case at the end of the period of total starvation each larva was supplied daily only with sufficient food to maintain a steady weight of 1.5–2.0 g. In contrast to the earlier results none of these larvae initiated metamorphosis: all underwent a supernumerary larval molt, apolysis taking place 5–11 days after resumption of restricted feeding.

In both series of experiments the supernumerary sixth instars always showed faulty development of crochets as well as the localized zones of pupal cuticle described earlier. Many died without further development and those which eventually initiated metamorphosis inevitably formed non-viable pupae showing misshapen head and wing structures.

These experiments again implicated the attainment of a weight near 3 g as critical for metamorphosis. Additional experiments employing variants of this starvation-refeeding scheme revealed no significant differences from those cited here: in every case the weight interval from 2.0–4.0 g demarcated a zone of transition from a population of larvae capable of larval molting to one uniformly initiating metamorphosis.

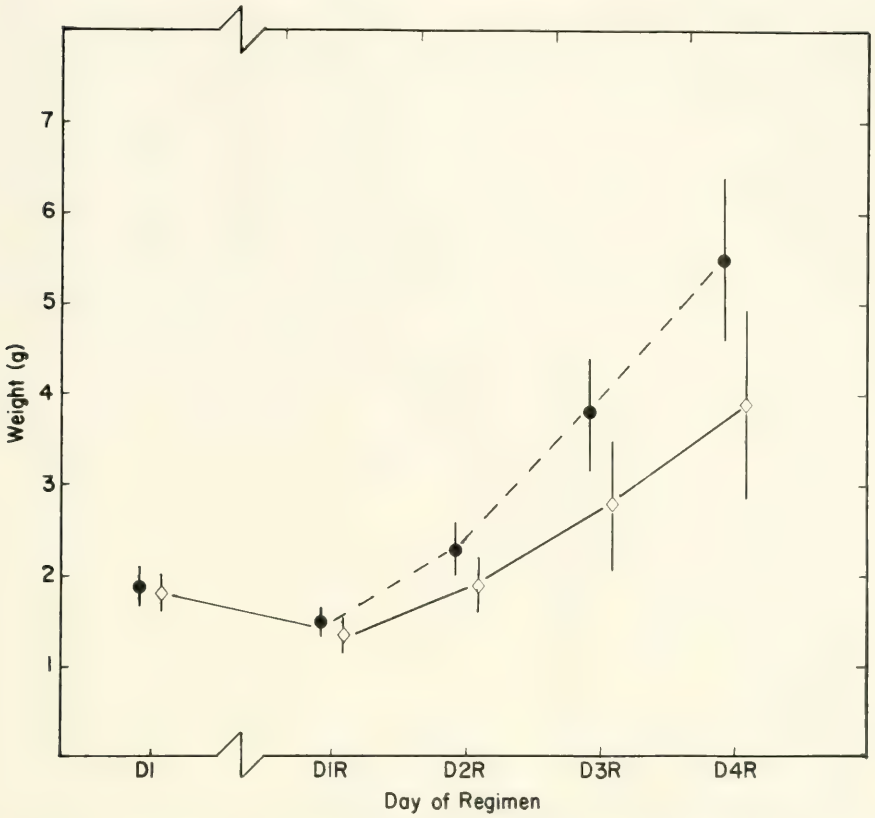

FIGURE 2. Relationship of fifth-instar larval growth rates under a starvation-refeeding protocol to the developmental outcome of the instar. The figure depicts the average weight ± S.D. of two groups of larvae on several days during the regimen. Larvae were removed from food during the last quarter of the first day of the instar (D1) and returned to food on Days 4–6 of the instar at about the same time of day (first day of refeeding for all groups: D1R). They were subsequently weighed daily during this same period. Twenty-two larvae (dark circles) exposed the dorsal vessel at weights of 5–9 g 96–120 h after resumption of feeding. Seven larvae (clear diamonds) molted to supernumerary sixth-instar larvae with head capsule slipping on Day 4 of refeeding.

Effects of malnourishment in the third and fourth instars

In a further series of experiments we removed fourth-instar SD larvae from food at weights from 0.30 to 0.90 g. They were starved during the remainder of the fourth instar except in the case of larvae under 0.60 g which were given small amounts of food to maintain them at approximately their initial weight so as to ensure their survival. All individuals were weighed again upon apolysis to the fifth instar. During the fifth instar they were fed *ad lib,* observed daily, and weighed at apolysis to a sixth instar or when dorsal vessel exposure signaled the onset of metamorphosis.

As shown in Figure 3, the weight at apolysis to the fifth instar was generally a strong predictor of the individual's fate at the conclusion of that instar. Larvae weighing more than 0.6 g almost without exception underwent metamorphosis, whereas those weighing less than 0.5 g more often underwent a supernumerary molt to a sixth instar. Figure 3 also depicts the weights at the end of the fifth instar. The vast majority of larvae initiating a molt to a sixth larval instar weighed less than 3.5 g and would

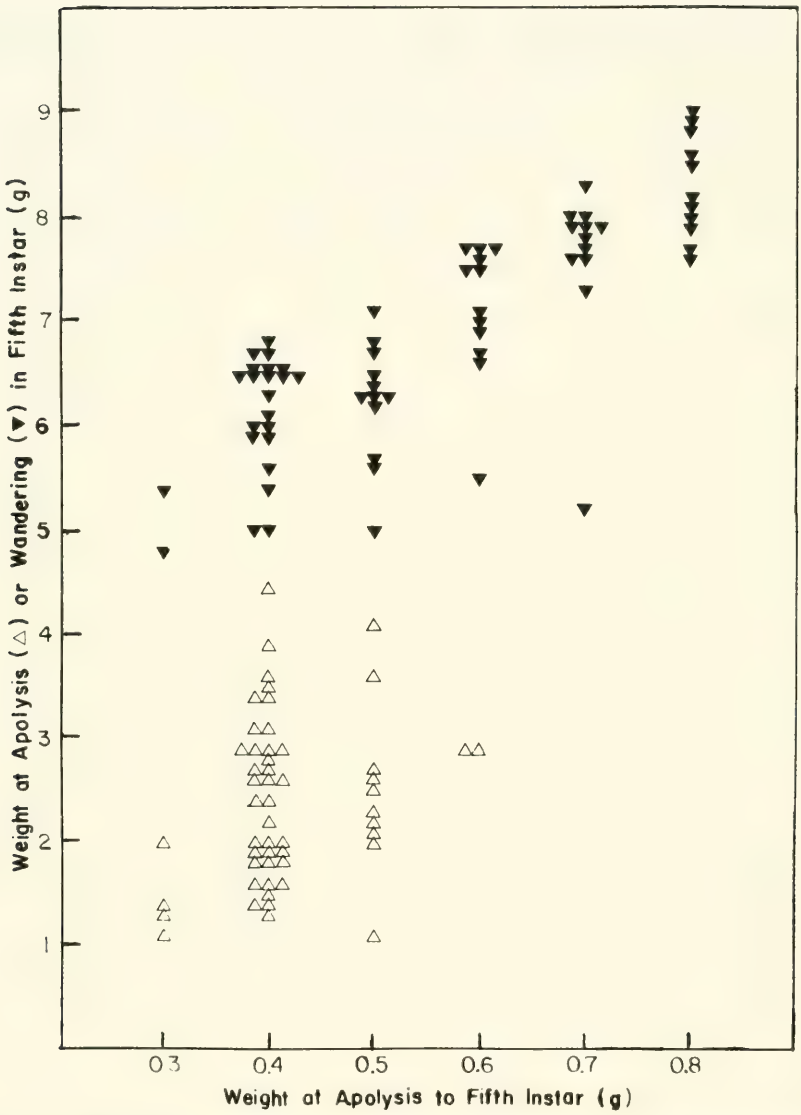

FIGURE 3. Transition from supernumerary larval molting to metamorphic development between 3 and 5 g. The live weight either at apolysis to the sixth instar (clear triangles) or at the time of initiation of the wandering period (dark triangles) is shown as a function of the same individual's weight at apolysis to the fifth instar. The range of sizes of the apolysing larvae was generated by partial starvation of the fourth instars. The weights recorded at the time of dorsal vessel exposure were undoubtedly lower than the maximum weight attained in the fifth instar by as much as 1 g due to the weight loss associated with gut evacuation and fluid loss at the outset of the wandering period.

have weighed approximately 0.5 g less at the time the molt was initiated about 12–18 h prior to apolysis (Trumen, 1972; Cymborowski *et al.*, 1982). Those weighing over 3.5 g nearly always underwent metamorphosis and with rare exception did so at weights over 5 g.

In a similar set of experiments third-instar SD larvae were held at 0.05–0.10 g until they molted to the fourth instar. These miniature fourths were then fed *ad lib* through both the fourth and fifth instars; their weights were subsequently recorded upon apolysis to the fifth instar and at apolysis or dorsal vessel exposure at the conclusion of the fifth instar.

Figure 4 depicts the fate of these larvae as a function of their weight at apolysis to the fifth instar. Similarities to the results of the experiments shown in Figure 3 are readily apparent. All larvae beginning the fifth instar above 0.6 g initiated metamorphosis, whereas those below 0.5 g underwent a supernumerary molt. Those undergoing a supernumerary molt did so at final weights under 2.0 g, whereas those initiating metamorphosis never weighed less than 6.0 g. The striking absence of

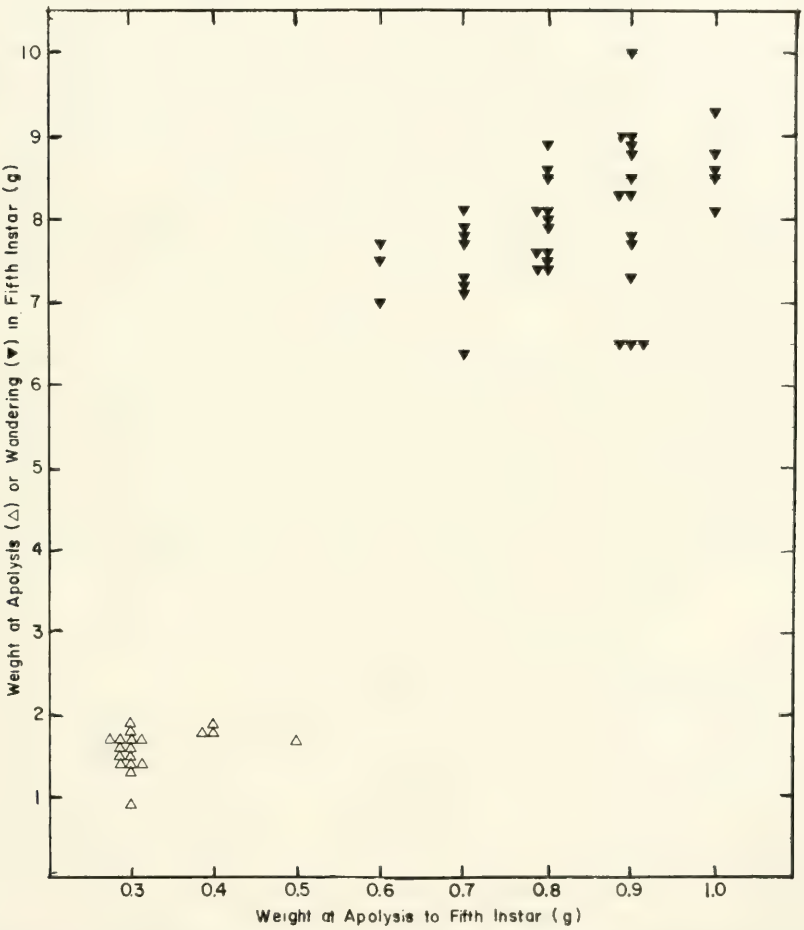

FIGURE 4. Transition from supernumerary larval molting to metamorphic development between 2 and 6 g. The live weight either at apolysis to the sixth instar (clear triangles) or at initiation of the wandering period (dark triangles) is shown as a function of the individual's weight at apolysis to the fifth instar. The range of sizes of the apolysing larvae was generated by prior partial starvation during the third instar. The weights recorded at the time of dorsal vessel exposure were undoubtedly lower than the maximum weight attained in the fifth instar by as much as 1 g due to the weight loss associated with gut evacuation and fluid loss at the outset of the wandering period.

development in the weight range of 2.0–6.0 g may be partly accounted for by a dearth of larvae entering the fifth instar between weights of 0.4–0.6 g—a finding encountered in the accompanying paper (Safranek and Williams, 1984).

In contrast to the aberrant supernumerary larvae resulting from starvation of normal fifth instars, the sixth instars formed from diminutive fifth-instar larvae appeared completely normal and eventually metamorphosed to pupae normal in appearance but for a slightly broader range of sizes.

These results pointed once again to the interval of 2–3 g as a critical threshold marking the transition from an endocrine milieu favoring a larval molt to one suitable for metamorphosis. But they also demonstrated that under some conditions a supernumerary molt could result in a normal sixth instar capable of normal metamorphosis and lacking the aberrations of crochets and imaginal discs noted in the sixths formed under the earlier regimens involving starvation of fifth instars.

Is the threshold relative or absolute?

In the experiments reported to this point, fifth-instar larvae underwent generalized metamorphosis only after attaining a weight of at least 2–3 g. In the accompanying paper (Safranek and Williams, 1984) we demonstrate that under normal culture conditions molting in earlier instars takes place only when a larva attains some multiple of its initial weight in that instar. We were curious as to whether the apparent threshold of 2–3 g represented a similar instance in which larvae were measuring their size relative to some standard set earlier in the instar or whether it represented an absolute threshold for the initiation of metamorphosis. This was examined in two sets of experiments.

In the first of these a group of fourth-instar SD larvae were held at a steady-weight of 0.65–0.75 g until they molted. A second group undergoing apolysis to the fifth instar was selected from stock in the weight range 1.3–1.6 g. Each of the two groups was further subdivided: some larvae were placed on a steady-weight regimen immediately upon ecdysis to the fifth instar; the others were fed and allowed to grow to some multiple of their initial weights before being placed on the steady-weight regimen. Table I records the results. In both groups the transition from larval to metamorphic development occurred at a maintenance weight of 2–2.2 g. Lower

TABLE I

Development of fifth-instar larvae held at various maintenance weights

Initial weight	Maintenance weight	Developmental fate*		
		Sixth-instar larvae	Larval-pupal intermediates	Pupae
0.65–0.75	1.3–1.5	12	0	0
1.3–1.5	1.3–1.5	12	0	0
0.65–0.75	1.6–1.9	12	2	0
0.65–0.75	2.0–2.2	0	7	5
1.3–1.6	2.0–2.2	3	5	4
0.65–0.75	2.7–3.0	0	0	12
1.3–1.6	2.7–3.0	0	1	11

* The number of larvae entering the several developmental pathways are indicated. Sixth-instar larvae had no traces of pupal cuticle except on their imaginal discs, where it appeared routinely. Larval-pupal intermediates presented a mosaic of larval and pupal cuticle. Pupae were without evidence of larval cuticle.

maintenance weights resulted only in supernumerary molting; higher weights, in metamorphosis.

In a second experiment fourth-instar SD larvae were subjected to a steady-weight regimen at weights of 0.4–0.5 g until they molted to the fifth instar, after which they were fed *ad lib.* The majority molted to sixth instars at weights from 1.6 to 3.5 g. These were then returned to steady-weight regimens either at their initial sixth-instar weight or after a brief period of feeding at a somewhat higher weight. Larvae maintained below 2.5 g almost always underwent a supernumerary molt whereas larvae above 3.0 g regularly metamorphosed (Fig. 5). Indeed, many of the larger larvae were able to undergo metamorphosis in the absence of any weight gain during the final instar.

In these two sets of experiments larvae molted to an additional larval instar when the molt was initiated below 2 g and underwent metamorphosis at weights beyond 3 g. This generalization held true even though larvae entered the nominally last instar over nearly a 3 g weight range from 0.65 to 3.5 g. Thus, the interval of 2–3 g appears to represent an absolute threshold whose passage is necessary and sufficient for metamorphosis. No evidence suggests that this passage occurs upon attainment of a multiple of the initial weight at the outset of the presumptive final instar.

Discussion

Two rules appear to govern the incidence and character of supernumerary molts by hornworm larvae under the feeding regimens employed here. First, an extra larval

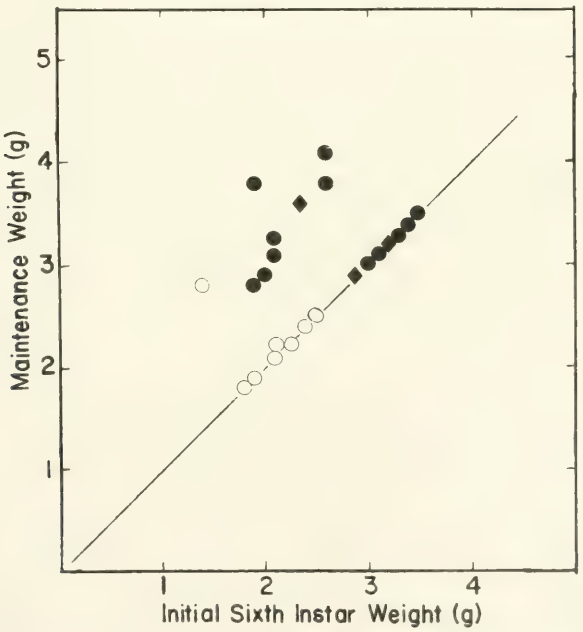

FIGURE 5. Developmental outcome of the sixth instar under a steady weight regimen at various maintenance weights as a function of the initial sixth-instar weight. Sixth-instar SD larvae were generated as described in Results and placed on a steady-weight regimen either at their initial body weight in that instar (data along solid line) or after an initial period of *ad lib* feeding (data above solid line). Larvae were observed daily for head-capsule slipping or dorsal vessel exposure, both of which occurred 10–28 days after initiation of the steady-weight regimen. After the molt individuals were judged to have formed a seventh larval instar (clear circles), a pupa (solid circles), or a larval-pupal intermediate (solid diamonds), the latter characterized by a mosaic pattern of larval- and pupal-like cuticle over the integument.

molt will be undertaken only if initiated at a weight below about 3 g; development initiated above 3 g follows a typical metamorphic sequence leading through the wandering period to pupation. Second, supernumerary sixth-instar larvae are morphologically aberrant and unable to pupate if they begin the preceding fifth instar at a weight above about 1 g.

The latter rule is supported by observations of fifth-instar larvae starved in the third or fourth instars. Those which entered the instar at a weight below 0.6 g regularly molted to sixths that were normal both in appearance and in their subsequent metamorphosis to pupae. These larvae respected both of the above rules: they initiated supernumerary molts at weights below 3 g and they entered the fifth instar at a weight below 1 g.

When, as under normal rearing conditions, *ad lib* feeding through the first four instars produced fifths weighing 1–1.5 g, no feeding protocol was then able to produce normal sixth instars: instead, the supernumerary larvae inevitably bore characteristic abnormalities reflecting the precocious metamorphosis of the crochets, the imaginal discs, and tissues about the head. These abnormalities were more pronounced as the supernumerary molt occurred at weights closer to 3 than to 1 g. Individuals so affected never metamorphosed to normal pupae. Prior investigations have disclosed a similar assortment of abnormalities in sixth-instar hornworms (Nijhout, 1976; Jones *et al.*, 1980; Cymborowski *et al.*, 1982). These results suggest the occurrence under normal circumstances of a developmental transition at the outset of the fifth instar which ensures the metamorphosis of the crochet epidermis and imaginal discs at the next molt without necessarily affecting the remainder of the integument.

The first of the above-mentioned rules describes a weight threshold for the induction of metamorphosis. This observation largely corroborates earlier work (Nijhout and Williams, 1974a) in which attention was drawn to the weight of 5 g: this was regarded as an approximate threshold size at which larvae could be starved and yet initiate metamorphosis synchronously with larvae fed *ad lib*—that is, without any delay. Here we direct attention to the acquisition of the lower weight between 2 and 3 g as an absolute prerequisite for the eventual metamorphosis of intact larvae. This threshold was most sharply defined in the experiments employing the steady-weight regimen in the final instar. A similar threshold could be discerned though less sharply after imposition of the steady-weight regimen in either the third or the fourth instars or under the starvation-refeeding schemes employed in the fifth instar in both this and prior studies (Jones *et al.*, 1980; Jones *et al.*, 1981; Cymborowski *et al.*, 1982). The apparent threshold defined by the latter regimens is likely to be higher and less well defined than that under a steady-weight regimen due to growth of the larva between the time the molt is initiated and the beginning of apolysis or between the time the threshold is crossed and the initiation of dorsal vessel exposure. For this reason, we consider the use of the steady-weight regimen in the normal final instar optimal for the determination of an accurate threshold size for metamorphosis.

Disparate frequencies of supernumerary molting in feeding-refeeding schemes have been reported in the several studies to date (Jones *et al.*, 1980; Jones *et al.*, 1981; Cymborowski *et al.*, 1982). Our results suggest that these differences may be attributed at least in part to the deployment of different photoperiods or to slight variations in the initial weight of fifth-instar larvae. Thus, in our experience SD larvae initiated metamorphosis at somewhat lower weights than LD larvae under otherwise identical regimens. Moreover, when larvae enter the fifth instar at weights around 0.7 g, starvation for 3 days followed by *ad lib* feeding always leads to a supernumerary larval molt (unpubl. obs.); by contrast, even 5 days of starvation failed to ensure larval molting of larvae entering the instar at 1–1.5 g in the experiments reported here.

Prior studies of the tobacco hornworm have emphasized the importance of the duration of malnutrition or of the composition of the food during the period of malnutrition. We are persuaded that the single major variable in regimens capable of eliciting a supernumerary molt is their ability to maintain fifth-instar larvae below the threshold of 2–3 g until the onset of a molt. Thus, full starvation of 1.5–2 g larvae for 3 days followed by maintenance on a steady-weight regimen at about 2 g always resulted in a supernumerary molt. By contrast, when similar larvae were allowed to feed *ad lib* after the 3 days of starvation, none underwent a supernumerary molt because they grew rapidly beyond the 3 g threshold for metamorphosis. Initial weight in the instar, weight at the time of starvation, and the duration of starvation are all likely to alter the incidence of supernumerary molting under any feeding regimen only insofar as they affect the size from which fifth-instar larvae undertake the subsequent molt.

The threshold of 3 g almost certainly marks the point of initiation of processes resulting in an irrevocable elimination of JH. Thus the hemolymph JH titer of normal larvae declines sharply beginning at 4–5 g—less than a day after reaching the threshold weight of 3 g (Nijhout and Williams, 1974b; unpubl. obs.). Furthermore the metamorphosis of the integument witnessed in larvae above 3 g can be largely prevented by topical administration of JH (Truman *et al.*, 1974). In normal larvae the loss of JH is likely to mirror accelerated breakdown of the hormone as well as its diminished production. The activity of JH esterase in both the hemolymph and tissues of fifth-instar *Manduca* increases beginning on Day 2 at about 3 g (Vince and Gilbert, 1977; Mitsui *et al.*, 1979; Beckage and Riddiford, 1981; unpubl. obs.). Moreover, a decline in CA activity has been noted about the same time (Bhaskaran *et al.*, 1980). The contribution of JH esterase to the metamorphosis of malnourished 3 g larvae in the current study was probably small: larvae of that size have relatively low levels of esterase even when fed *ad lib* and starvation typically induces a marked decline of prevailing esterase levels (Sparks *et al.*, 1983; unpubl. obs.). In all likelihood the present results reflect the onset of a decline of CA activity at a weight of about 3 g. In this regard we recall the suggestion of Bhaskaran *et al.* (1980) that a neurohumoral brain-derived factor released at a weight of approximately 3 g can curtail the endocrine activity of the CA.

Prior investigators of supernumerary molting induced by starvation have regarded the entire phenomenon as largely dependent on an atypical pattern of regulation of the CA and the JH titer (Bhaskaran and Jones, 1980; Cymborowski *et al.*, 1982). Data on the JH titer of starved fifth-instar larvae destined for a sixth instar indicate the level to be high during the period of starvation at 1 g and then to decline rapidly once feeding is resumed and the larvae approach weights of 3–4 g (Nijhout, 1975b; Cymborowski *et al.*, 1982). But this picture in fact resembles that of normal fifth instars where JH titers are initially quite high (Fain and Riddiford, 1975; Nijhout and Williams, 1974b). Although these elevated titers begin to decline shortly after ecdysis to the fifth instar, JH normally remains detectable at levels sufficient to block metamorphosis up to a weight of at least 5 g (Nijhout and Williams, 1974b; Nijhout, 1976). Since the vast majority of the hundreds of supernumerary molts observed in the course of the present study occurred at weights less than 3 g, the presence of JH at levels sufficient to ensure larval molting is hardly exceptional and is at best a precondition for supernumerary molting. Starvation-induced supernumerary molts reflect in considerable measure the ability of these feeding regimens to hold larvae at the low weights characteristic of the early final instar when JH levels are typically high. But the critical effect of starvation is to distort the usual size parameters associated with development in the final instar so that a molting gestalt is achieved at the low weights normally associated with high JH levels.

As we point out in the accompanying paper, starvation permits the achievement of a molting gestalt at much lower weights than occurs under conditions of normal feeding. In the fifth instar, as in earlier instars, starvation apparently short-circuits the normal machinery which links the onset of ecdysone-dependent development to the attainment of a critical size even to the extent of permitting larval molting in the absence of any growth during the instar. This could happen through several mechanisms: starvation may actively induce the endocrine changes that lead to accumulation of a molt-inducing titer of ecdysone or it may simply permit the passage of time to accomplish gradually what is completed promptly under conditions of normal growth. Different regimens can variously affect the duration of the instar prior to the onset of molting. Thus, fifth instars held at about 1 g initiated supernumerary molting after a minimum of 16 days and an average of nearly 3 weeks; by contrast, similar individuals totally starved for 5 days after ecdysis and then fed *ad lib* molted on either Day 8 or 9. Whatever the mechanism, this downward adjustment in the weight at which ecdysone-dependent development ensues has been noted after starvation in each instar examined (Safranek and Williams, 1984) and is necessary for the formation of a supernumerary instar by fifth-instar hornworms.

Supernumerary larval molting can be understood as the outcome of two facets of the hornworm's endocrine physiology: (1) the normal presence in all hornworms less than about 3 g of sufficient JH to permit a larval molt, and (2) the downward adjustment induced by starvation in the weight at which ecdysone-dependent development begins. When a large downward adjustment leads fifth-instar larvae to molt at weights below the critical threshold of 3 g, a supernumerary molt ensues; smaller adjustments result in the initiation of development at weights above this threshold where the JH level is too low to permit additional larval development, thus assuring metamophosis. This model suggests that a feeding regimen will tend to generate supernumerary larval molting to the degree that it can slow the growth rate and/or accelerate the generation of a molting gestalt in fifth-instar larvae. In this view, malnutrition does not bring about a supernumerary larval molt primarily through peculiar effects on the JH titer; rather, it serves to fix the individual in an endocrine milieu appropriate for additional larval development until changes in ecdysone production, metabolism, or sensitivity initiate a molt.

ACKNOWLEDGMENTS

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LITERATURE CITED

BECKAGE, N. E., AND L. M. RIDDIFORD. 1982. Effects of parasitism by *Apanteles congregatus* on the endocrine physiology of the tobacco hornworm *Manduca sexta*. *Gen. Comp. Endocrinol.* **47**: 308–322.

BELL, R. A., AND F. G. JOACHIM. 1976. Techniques for rearing laboratory colonies of tobacco hornworms and pink bollworms. *Ann. Entomol. Soc. Am.* **69**: 365–373.

BHASKARAN, G., AND G. JONES. 1980. Neuroendocrine regulation of corpus allatum activity in *Manduca sexta:* The endocrine basis for starvation-induced supernumerary larval moult. *J. Insect Physiol.* **26**: 431–440.

BHASKARAN, G., G. JONES, AND D. JONES. 1980. Neuroendocrine regulation of corpus allatum activity

in *Manduca sexta:* Sequential neurohormonal and nervous inhibition in the last-instar larva. *Proc. Natl. Acad. Sci. USA* **77:** 4407–4411.

CYMBOROWSKI, B., M. BOGUS, N. E. BECKAGE, C. M. WILLIAMS, AND L. M. RIDDIFORD. 1982. Juvenile hormone titres and metabolism during starvation-induced supernumerary larval moulting of the tobacco hornworm, *Manduca sexta* L. *J. Insect Physiol.* **28:** 129–135.

FAIN, M. J., AND L. M. RIDDIFORD. 1975. Juvenile hormone titers in the hemolymph during late larval development of the tobacco hornworm, *Manduca sexta* (L). *Biol. Bull.* **149:** 506–521.

JONES, D., G. JONES, AND G. BHASKARAN. 1980. Induction of supernumerary molting by starvation in *Manduca sexta* larvae. *Ent. Exp. Appl.* **28:** 259–267.

JONES, D., G. JONES, AND G. BHASKARAN. 1981. Dietary sugars, hemolymph trehalose levels, and supernumerary molting of *Manduca sexta* larvae. *Physiol. Zool.* **54:** 260–266.

MITSUI, T., L. M. RIDDIFORD, AND G. BELLAMY. 1979. Metabolism of juvenile hormone by the epidermis of the tobacco hornworm, *Manduca sexta. Insect Biochem.* **9:** 637–643.

NIJHOUT, H. F. 1975a. A threshold size for metamorphosis in the tobacco hornworm, *Manduca sexta* (L.). *Biol. Bull.* **149:** 214–225.

NIJHOUT, H. F. 1975b. Dynamics of juvenile hormone action in larvae of the tobacco hornworm, *Manduca sexta* (L.). *Biol. Bull.* **149:** 568–579.

NIJHOUT, H. F. 1976. The role of ecdysone in pupation of *Manduca sexta. J. Insect Physiol.* **22:** 453–463.

NIJHOUT, H. F., AND C. M. WILLIAMS. 1974a. Control of moulting and metamorphosis in the tobacco hornworm, *Manduca sexta* (L.): growth of the last-instar larva and the decision to pupate. *J. Exp. Biol.* **61:** 481–491.

NIJHOUT, H. F., AND C. M. WILLIAMS. 1974b. Control of moulting and metamorphosis in the tobacco hornworm, *Manduca sexta* (L.): cessation of juvenile hormone secretion as a trigger for pupation. *J. Exp. Biol.* **61:** 493–501.

RIDDIFORD, L. M., AND A. T. CURTIS. 1978. Hormonal control of epidermal detachment during the final feeding stage of the tobacco hornworm larva. *J. Insect Physiol.* **24:** 561–568.

SAFRANEK, L., AND C. M. WILLIAMS. 1984. Determinants of larval molt initiation in the tobacco hornworm, *Manduca sexta. Biol. Bull.* **167:** 568–578.

SPARKS, T. C., B. D. HAMMOCK, AND L. M. RIDDIFORD. 1983. The haemolymph juvenile hormone esterase of *Manduca sexta* (L.)—inhibition and regulation. *Insect Biochem.* **13:** 529–541.

TRUMAN, J. W. 1972. Physiology of insect rhythms. I. Circadian organization of the endocrine events underlying the moulting cycle of larval tobacco hornworms. *J. Exp. Biol.* **57:** 805–820.

TRUMAN, J. W., AND L. M. RIDDIFORD. 1974. Physiology of insect rhythms. III. The temporal organization of the endocrine events underlying pupation of the tobacco hornworm. *J. Exp. Biol.* **60:** 371–382.

TRUMAN, J. W., L. M. RIDDIFORD, AND L. SAFRANEK. 1974. Temporal patterns of response to ecdysone and juvenile hormone in the epidermis of the tobacco hornworm, *Manduca sexta. Dev. Biol.* **39:** 247–262.

VINCE, R. K., AND L. I. GILBERT. 1977. Juvenile hormone esterase activity in precisely timed last instar larvae and pharate pupae of *Manduca sexta. Insect Biochem.* **7:** 115–120.

Reference: *Biol. Bull.* **167**: 568–578. (December, 1984)

DETERMINANTS OF LARVAL MOLT INITIATION IN THE TOBACCO HORNWORM, *MANDUCA SEXTA*

LOUIS SAFRANEK AND CARROLL M. WILLIAMS

The Biological Laboratories, Harvard University, Cambridge, Massachusetts 02138

ABSTRACT

Hornworm larvae adhere to Dyar's rule under normal growth conditions, increasing their live weight by an average of 5- to 6-fold from the outset of one instar to the next. This adherence to Dyar's rule is largely maintained even in instars subsequent to those in which larvae have been severely malnourished. The importance of relative weight gain for the normal onset of larval molting contrasts with the requirement for attainment of an absolute body weight prior to pupation. But body size is by no means the sole cue for the initiation of a larval molt. Thus, under conditions of malnutrition larvae can initiate a molt at any weight and can do so even in the absence of any weight gain in an instar: in these circumstances the duration of an instar is inversely related to the weight at the outset of malnutrition. In larvae fed *ad lib* as well as in malnourished larvae the initiation of a molt appears to be limited to a discrete phase of the photocycle. The failure of larvae to molt on schedule at body weights lower than those predicted by Dyar's rule cannot be attributed to an inability of the brain to stimulate ecdysone-dependent development as is the case in diapausing pupae. Though body size, instar duration, and photocycle all interact to determine the onset of the molt, the role of the brain remains ill-defined.

INTRODUCTION

Entomologist Harrison Dyar achieved a measure of lasting fame with his three-page 1890 paper reporting that the width of the head capsule of larval Lepidoptera increased from molt to molt by a factor that was generally constant for a given species—an empirical relationship that came to be known as "Dyar's law" or "Dyar's rule." Subsequent work suggested that every dimension of a larva progresses from one instar to the equivalent stage in the next by a constant factor, and so too does the larval weight (Bodenheimer, 1933). The rule was originally promulgated as a means for identifying successive instars, but its usefulness in this arena as well as its general validity have been repeatedly debated (for review, see Beck, 1950). Exceptions to the rule are abundant yet, beginning with Dyar's own observations, studies have repeatedly confirmed the applicability of the rule to at least some species under appropriate growth conditions. Well-documented examples include the cabbage looper *Trichoplusia ni* (Jones *et al.*, 1981) and the tobacco hornworm *Manduca sexta* (Nijhout, 1975)—the latter the subject of the present paper.

Even in instances where Dyar's rule cannot be rigorously applied, a direct relationship between growth and the initiation of molting is typically apparent. But the actual mechanism by which larvae gauge their mass remains an enigma. Only in the two instances of the hemipterans *Rhodnius prolixus* and *Oncopeltus fasciatus* is an important relationship apparent between physical distention *per se* and the initiation

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Abbreviations: LD, long day; SD, short day.

of molting (Beckel and Friend, 1964; Blakley and Goodner, 1978; Nijhout, 1979)—a link that may well be mediated by distention-induced activation of neural stretch receptors. In other instances including the tobacco hornworm our fragmentary understanding allows us to approach the functional circuitry only as a black box. To facilitate discussion we propose to name this unknown mechanism linking the attainment of a particular body size to the initiation of a molt the "Dyarstat."

Under normal feeding conditions the tobacco hornworm exhibits a remarkable adherence to Dyar's rule. This was first appreciated by Nijhout (1975) who showed that the live weight of the larval population increased between equivalent stages in successive instars by a factor of about 5.7 through the first four of the hornworm's five instars. Thus, the hornworm's Dyarstat can punctuate the typical course of larval exponential growth with molts at precise intervals. The present study focuses on the play of the Dyarstat under various feeding conditions and examines as well the contributions of growth, instar duration, and photocycle to the initiation of the molt. Though the Dyarstat exhibits a remarkable stability under many conditions, larval size proves to be only one of several factors that predictably shape the pattern of larval molting.

Materials and Methods

Hornworms were reared at 25°C on an artificial diet as described by Truman (1972) and Bell and Joachim (1976) under either short-day (SD, 12L:12D) or long-day (LD, 17L:7D) photoperiods. Under these conditions the larvae routinely underwent five larval instars. Time-of-day was arbitrarily referenced to lights-off at midnight (24:00 = 00:00). The timing of events in the life cycle was as described by Truman (1972) and Truman and Riddiford (1974) except that we have termed the first 24 h of each instar Day 1 rather than Day 0.

In a feeding protocol described as the "steady-weight regimen" larvae were weighed and returned to individual, clean, capped plastic containers which contained no food. Thereafter at daily intervals each individual was weighed and provided with an amount of diet sufficient to restore it to near its original weight. This regimen was maintained until molting was initiated, as signaled by apolysis of the head capsule.

Removal of larval and pupal brains and implantation of brains into debrained diapausing pupae was accomplished as previously described (Safranek and Williams, 1980).

Results

Comparative development of first- and second-gate fourth-instar larvae

In the experiments described here, groups of SD pharate fourth-instar larvae were selected from our stock at the outset of the photophase. All these larvae underwent ecdysis within the next 12 h. We term this group of larvae beginning the fourth instar on the same day a cohort. Within a cohort the covert head-dependent events which initiate the molt to the fifth instar typically occur during the scotophase of either the third or fourth day of the instar (Truman, 1972). The overt initiation of the molt signaled by head capsule slipping ordinarily ensues half a day later during the photophase of the same day. In these experiments fourth instars of various ages were removed from their containers, weighed, and then returned to their food and observed for the onset of head capsule slipping. Larvae which underwent slipping on Day 3 of the instar were termed "first-gate larvae;" those which did so on Day 4, "second-gate larvae."

In an examination of over a thousand pharate fourth-instar larvae studied in cohorts of 50–100 over the past several years, more than 95% weighed 0.16 to 0.23 g, the average ranging from 0.18 to 0.20 g. First-gate and second-gate larvae were indistinguishable on the basis of their weights as pharate fourths; nevertheless larvae destined to be first-gate could be roughly separated from those of the second-gate by the hour of ecdysis to the fourth instar, the former tending to complete the molt earlier in the day by an average of several hours. By the outset of the photophase on the third day of the instar, weight could largely be relied on to distinguish these two groups. Thus, as illustrated in Figure 1, first-gate larvae appeared more frequently among the larger Day 3 larvae. Indeed, all individuals weighing at least 0.9 g initiated apolysis later on Day 3 whereas all but a few weighing 0.6 g or less waited until Day 4. Nearly twice as many first-gate as second-gate larvae were among the 246 individuals in this experiment; their average weights on the morning of Day 3 were 1.02 ± 0.17 g and 0.68 ± 0.13 g, respectively.

Although first-gate fourths are larger than their second-gate counterparts on the morning of Day 3, their final size at the time of apolysis to the fifth instar is typically less than that of second-gate larvae. The latter, having failed to initiate the molt during the gate on Day 3, are obliged to await the arrival of the gate on Day 4. As a result, the duration of the feeding period from ecdysis to the fourth instar until apolysis to the fifth instar is typically 15–18 h longer for second-gate than for first-gate larvae. This largely accounts for the 0.1 to 0.2 g average weight difference that may be seen between mature first- and second-gate fourths within a given cohort.

From data on the weight of several hundred larvae as pharate fourth and fifth instars we calculated a constant after the manner of Dyar—namely the quotient of the average weight at apolysis to the fifth instar divided by that at apolysis to the

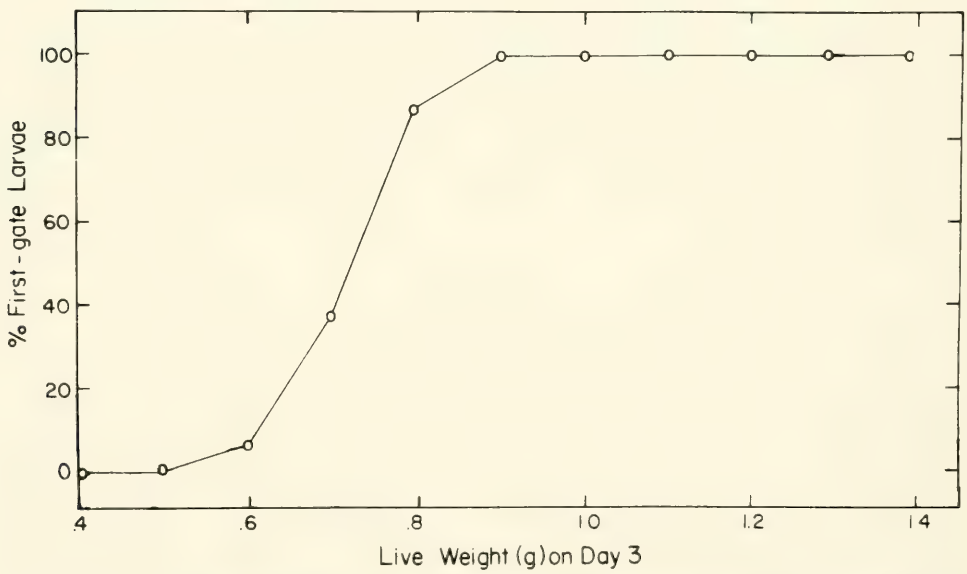

FIGURE 1. Weight-dependence of molt initiation by first-gate fourth-instar larvae. Fourth-instar SD larvae were weighed at the outset of the photophase (12:00) on Day 3 of the fourth instar and were considered first-gate larvae if head capsule slipping was apparent 24 h later. Each point represents the percentage of first-gate larvae in an interval extending 0.1 g above the corresponding weight on the x-axis. The number of larvae per category ranged from 5 to 10 at the extremes to 30–40 over the range 0.6–1.0 g.

fourth. The calculated value of 5.8 was in remarkable agreement with the value of 5.7 found by Nijhout (1975). The individual values ranged from 4.4 to 8.4, with larger values predominanting among second-gate larvae.

Effects of a steady-weight feeding regimen on the development of fourth- and fifth-instar larvae

SD fourth-instar larvae were divided into groups of 10–25 larvae at 0.1 g intervals between 0.15 g and 0.95 g and placed on a steady-weight regimen as described under Materials and Methods, daily receiving sufficient food to restore them to within .03 g of their initial weights. All survived to molt to the fifth instar except for 6 of 25 individuals placed on the regimen immediately after ecdysis to the fourth instar; these died without further development. Though many larvae molting at weights below 0.4 g failed to ecdyse, the old cuticle could readily be peeled away revealing in nearly all cases the presence of an intact fifth-instar larval cuticle. As shown in Figure 2, the duration of the instar was inversely related to the weight at the initiation of the steady-weight regimen. The instar was significantly prolonged for all groups of larvae

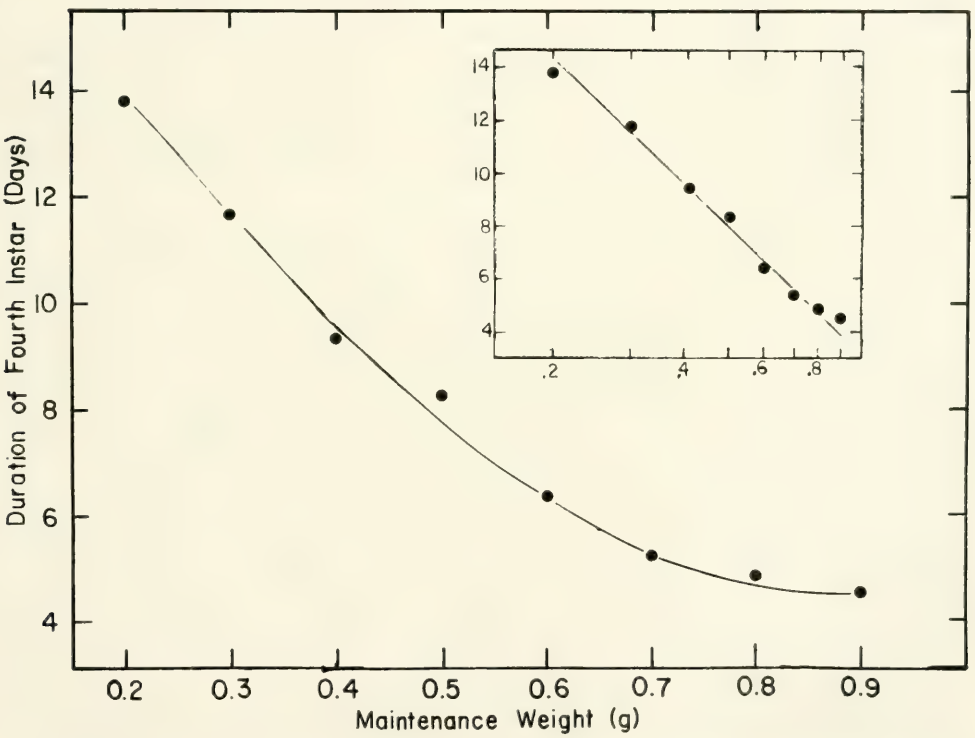

FIGURE 2. Inverse relationship between instar duration and the maintenance weight under a steady-weight regimen. Fourth-instar SD larvae weighing from 0.15 to 0.95 g were maintained on a steady-weight regimen as described under Materials and Methods and Results. The figure plots the duration of the instar from the time of apolysis to the fourth instar to the time of apolysis to the fifth instar as a function of the maintenance steady-weight. The standard deviation decreased steadily from 3 days at 0.2 g to 0 at 0.9 g. Each point represents the average instar duration for 10 larvae except for the point at 0.2 g which represents 19 larvae. Each point represents an interval extending ± 0.05 g about the corresponding weight on the x-axis. The inset is a semilog plot of the same data.

except those maintained at weights above 0.65, most of which molted without a delay relative to similar larvae fed *ad lib.* When examined on a semilog plot, the data approximate a straight line.

A similar experiment was performed on 25 newly ecdysed SD fifth-instar larvae weighing from 0.8 to 1.2 g which were maintained by daily feedings within 0.05 g of their initial weights. In a fashion similar to the fourth instar, all individuals underwent apolysis, in this instance, after 16–23 days. They uniformly failed to ecdyse. Attempts to salvage several of them by manually removing the old cuticle proved to no avail since they failed to resume feeding. In every case a supernumerary sixth-instar larva had formed.

Development of larvae after malnourishment in the third or fourth instars

In the above experiments we found that under regimens of *ad lib* feeding larvae initiated the molt in a weight-dependent fashion after a weight increase of at least 4-fold; but under regimens of severe malnourishment they could molt after little or no weight gain within an instar. In additional experiments we examined the pattern of growth and molting in instars subsequent to those in which larvae had experienced severe malnutrition.

To this end, third-instar larvae weighing between 0.05 and 0.10 g were removed from diet and then maintained within this weight interval by small daily feedings; these weights correspond to about 25–50% of the maximum size typically attained by third instars fed *ad lib.* Larvae were weighed at the time of apolysis at the end of the third instar and daily thereafter. They were fed *ad lib* from the time of ecdysis to the fourth instar until the onset of the wandering period at the end of the fifth or sixth instar.

Figure 3 depicts the weight distribution of larvae at the outset of the fourth and fifth instars as well as the developmental fate at the end of the fifth instar for larvae from each 0.01 g division. As can be seen, larval weights were initially distributed more or less evenly. By contrast, larvae exhibited a bimodal distribution at apolysis to the fifth instar; by that time they had segregated into distinct weight categories which forecast their subsequent development. Thus, all larvae below 0.5 g underwent a supernumerary larval molt at the end of the fifth instar, while all those above this weight initiated metamorphosis. The two groups exhibited very different Dyar's ratios in the fourth instar, those with a subsequent supernumerary molt having an average ratio of 4.8, those metamorphosing at the end of the fifth instar an average ratio of 10.3. Though these two groups were separated by about 0.2 g at the end of the fourth instar, they became widely separated in weight prior to the onset of development at the end of the fifth instar: all fifths undergoing a supernumerary molt did so at weights below 2.5 g, whereas those that underwent metamorphosis weighed over 6 g at the time of dorsal vessel exposure. The growth ratio from the fifth to the sixth instar for larvae undergoing a supernumerary molt averaged 4.3.

In a subsequent experiment we examined the development of SD larvae in the fifth instar after they had been subjected to a steady-weight regimen in the fourth instar at maintenance weights from 0.4 to 0.9 g. In this instance larvae that were fed *ad lib* after molting to the fifth instar at weights above 0.6 g metamorphosed at the conclusion of the fifth instar at weights above 6 g. Larvae fed *ad lib* after entering the fifth instar at 0.4 to 0.6 g either underwent metamorphosis as fifths at weights above 5 g or underwent a molt to supernumerary sixth instars at weights from 1.1 to 4.5 g (mean: 2.5 g). Larvae undergoing a supernumerary molt exhibited an average Dyar's ratio of 5.2.

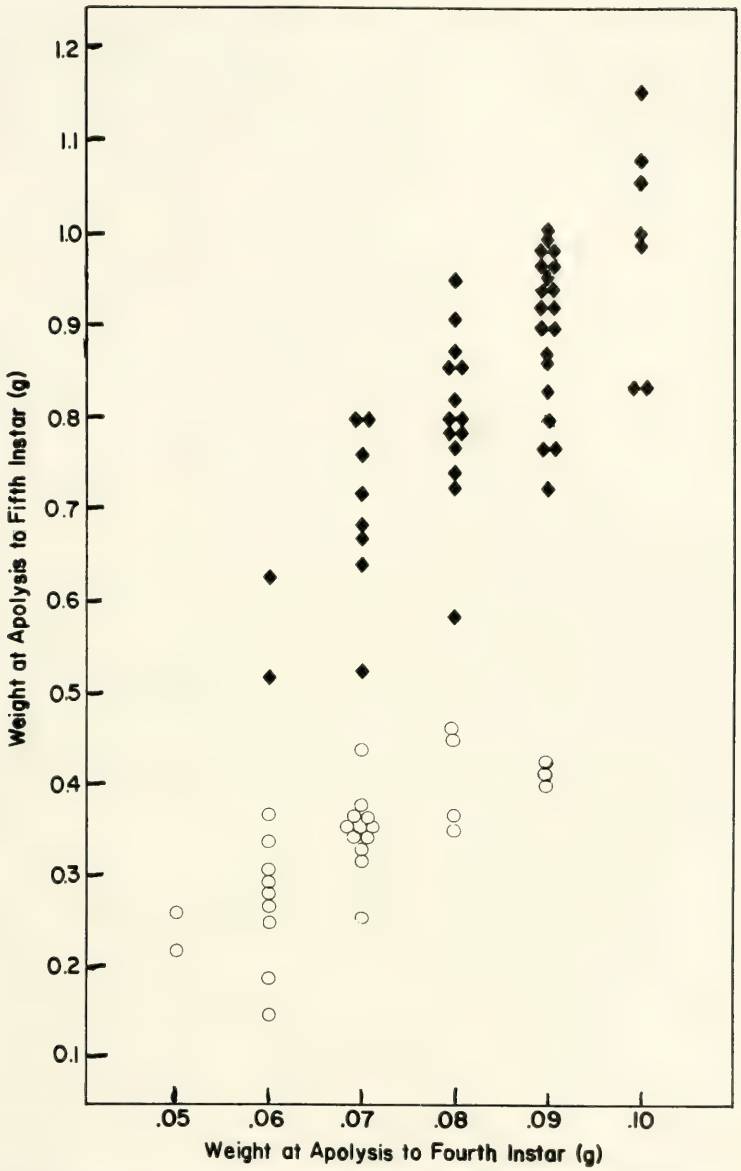

FIGURE 3. Weight of larvae at the time of apolysis to the fifth instar as a function of the weight at the time of apolysis to the fourth instar. Larvae were maintained on steady-weight regimens during the third-instar and fed *ad lib* thereafter. The duration of the fourth-instar for larvae initiating metamorphosis at the end of the fifth instar (solid diamonds) was typically one day longer than that for those molting to a supernumerary larval instar at the end of the fifth (clear circles). This accounts for their significantly greater proportionate weight increase in the fourth instar; but careful analysis of the growth data through the fourth instar fail to suggest a reason for this additional day of growth.

Is molting by malnourished larvae gated by the photocycle?

We inquired whether the initiation of larval molting at the conclusion of the fourth instar was coupled to the photocycle under conditions of malnutrition as it

is under *ad lib* feeding (Truman, 1972). To this end we recorded the onset of head capsule apolysis in groups of normally fed first-gate (n = 129) and second-gate (n = 250) SD larvae and in a group of SD larvae maintained on a steady-weight regimen at weights between 0.4 and 0.5 g (n = 60). Figure 4 depicts the percentage of larvae initiating head-capsule slipping in different phases of the 24 h photocycle. Eighty

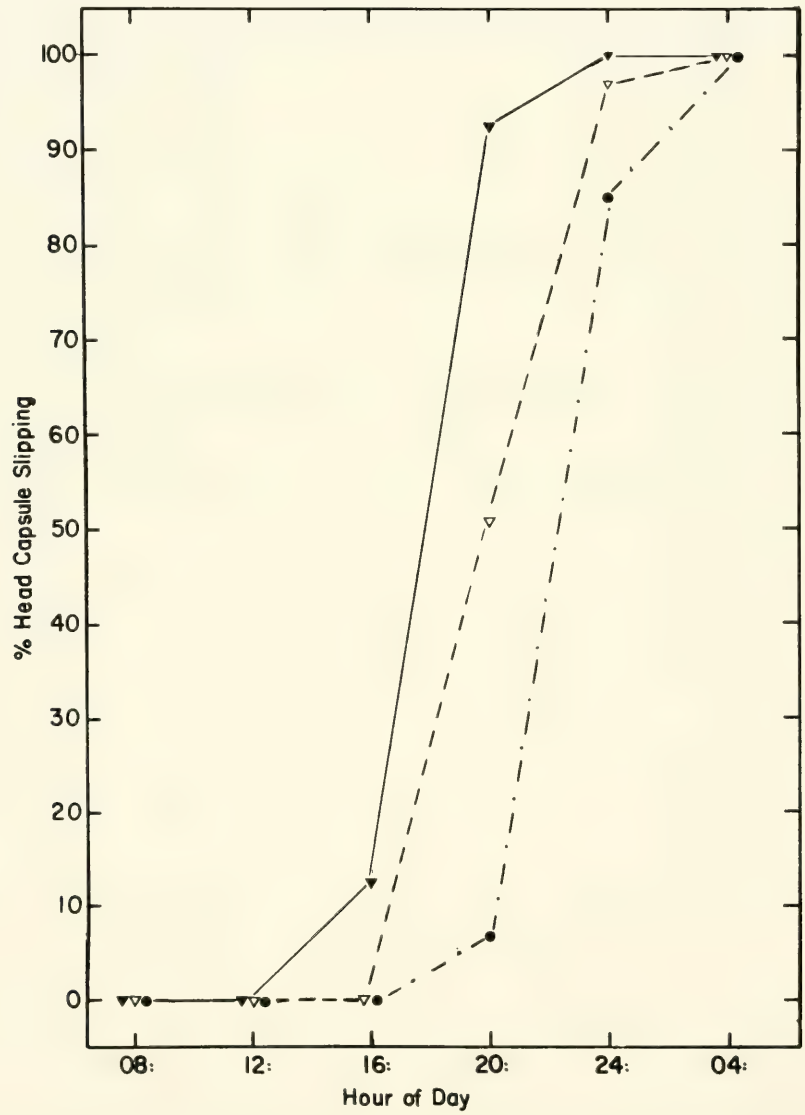

FIGURE 4. Cumulative percentages of first-gate (clear triangles), second-gate (dark triangles), and malnourished larvae (dark circles) initiating head capsule slipping at 4 h intervals at the end of the fourth instar. The day indicated on the abscissa extends from Day 3 to Day 4 of the fourth instar for first-gate larvae and from Day 4 to Day 5 for second-gate larvae. Malnourished larvae were observed at 4 h intervals during the several days over which they underwent apolysis: the data presented are derived by collapsing the observations of these several days into one. Larvae were observed under red light during the scotophase. The hour of the day was referenced to lights-off at midnight (24:00 = 00:00).

percent of second-gate larvae slipped their head capsules during the middle third of the photophase; first-gate larvae often did so during its final third. The majority of starved larvae also slipped their head capsules during the final third of the photophase, though some delayed until the ensuing scotophase. Thus, although the time of head-capsule slipping is shifted to the last portion of the photophase or slightly later, initiation of molting apparently remains closely coupled to the photocycle even in severely malnourished larvae.

Assay of ecdysiotropic activity of larval brains

At the outset of the fourth-instar larvae on an *ad lib* feeding regimen are 3–4 days from the initiation of the next molt. As we have seen, malnutrition early in this period can delay the onset of molting for as long as two weeks. We inquired as to whether the ecdysiotropic activity of the brain during the early fourth and fifth instars was curtailed as in the case of the diapausing pupal brain (Safranek and Williams, 1980). To this end we removed the brains from normally fed as well as malnourished larvae during the fourth or fifth instars. As further controls, we bioassayed brains excised from newly ecdysed diapause and non-diapausing pupae. The ecdysiotropic activity of each brain was assayed by implanting it into the head of a one-day-old diapause-destined pupa whose own brain had been simultaneously excised. The assay pupae were then examined daily for initiation of development as signaled by tracheal apolysis in the wings.

As summarized in Table I, brains from every group of larvae provoked the initiation of adult development by the assay pupae at similar rates and resembled in this regard the isolated brains of non-diapausing pupae. All were clearly more active than the brains of diapause-destined pupae. Manifestly, the larval brains even from malnourished larvae early in the fourth instar were not without significant ecdysiotropic

TABLE I

Ecdysiotropic activity of larval hornworm brains in a pupal assay

Stage of donor	Mean day of tracheal apolysis
Fourth instar, Day 1	10
Fourth instar, Day 2	9
Fourth instar, Day 3	12
Fourth instar, Day 4	10
Fourth instar, Day 5	11
Fifth instar, Day 1	11
Fourth instar, steady-weight	11
LD pupa, Day 1	8
SD pupa, Day 1	73

Brains were removed from SD larvae or from LD or SD pupae at the several stages shown and were implanted singly into brainless diapause-destined pupae less than one-day-old. Assay pupae were examined daily thereafter for tracheal apolysis. The mean value is the average day on which tracheal apolysis occurred in each group of 10–12 assay pupae. Standard deviations ranged between one and two days for all groups except SD pupae which had a deviation of four weeks. The operations were performed during the last six hours of the photophase on the day indicated. Day 3 fourths were all second-gate larvae which would have initiated the molt during the ensuing scotophase on Day 4. Day 4 fourths were also second-gate larvae which had just initiated head capsule slipping. Day 5 fourths were actually pharate fifth instars several hours prior to ecdysis. Steady-weight fourth instars were removed from diet at weights between .40 and .50 g on Day 2 and placed on a steady-weight regimen: their brains were removed for assay on Day 4, several days prior to the time when similar unoperated larvae underwent apolysis.

activity. These data suggest that the developmental delay witnessed in malnourished larvae is not due to the exercise of a diapause-like program by the brain.

Discussion

Exponential growth of hornworm larvae is punctuated by molts in a remarkably regular pattern: in each of the first four instars the average weight under normal conditions increases between molts by a constant factor. The constant of 5.7 described in Nijhout's detailed original study (Nijhout, 1975) continues very nearly to characterize our present laboratory stock, which exhibited a factor averaging between 5.5 and 6 in the present study of fourth-instar hornworms.

Within the constancy exhibited by the population as a whole, the Dyar's factor of individual fourth-instar hornworms varied considerably. This apparently stemmed largely from two sources. Because the initiation of molting is limited to a certain portion of the photocycle, larvae which attain a weight appropriate for molting continue to feed and grow until the permissive interval or "gate" arrives. In the case of fourth-instar larvae this period of growth may vary from 0 to at least 12 h and thus might have been responsible for weight differences of 0.25 g or more at the time of molting. A second source of variation is the approximately 18 h interval which routinely elapses from the point at which induction of the molt no longer requires the presence of the head until the onset of head capsule apolysis. In our experience this interval is of nearly constant duration regardless of whether larvae are feeding or being starved: consequently, any variations in the rate of growth at this time are not compensated by a change in the duration of feeding and so have significant effects on the ultimate size attained.

The present studies permit definition of a more reliable developmental threshold for larval molting. Small first-gate fourth-instar larvae on the morning of Day 3 are likely to be of a weight just adequate to have permitted the head-dependent events that initiate a molt to be completed during the gate on the preceding night. In the experiments here (Fig. 1) the smallest first-gate larvae at this time weighed 0.7–0.8 g—about 4 times as much as at the outset of the instar. In the further experiment summarized in Figure 2, only larvae weighing at least 0.7–0.8 g regularly initiated a molt without delay when further growth was prevented by starvation. Thus, once again acquisition of a weight about four times that at the outset of the instar appeared adequate for the initiation of molting at the ensuing gate. These experiments suggest that under conditions of *ad lib* feeding a four-fold increase in body weight prior to the end of the photocyclic gate is necessary for the completion of those head-centered events necessary for the induction of molting in the normal fourth instar.

When periods of malnutrition in the third or fourth instars forced larvae to molt at lower than normal weights, the Dyar's factors calculated for subsequent instars were generally between 4 and 5.5. These were slightly lower than the population average of normal larvae but near the average of first-gate larvae. Whether the majority of these larvae were effectively first-gate larvae or whether in fact the Dyarstat had been reset to a slightly lower weight range we cannot say.

Although molt initiation was a function of size under *ad lib* feeding conditions, under conditions of malnutrition molting could occur at any weight when sufficient time was allowed. Indeed, larvae in both the fourth and fifth instars proved able to initiate a molt in the absence of any increase from their weight at the time of ecdysis. Dyar (1890) suggested that the regular saltatory growth which he witnessed in the size of the head capsule could be used to identify successive larval instars. Although this system can be successfully applied to the hornworm under optimal feeding con-

ditions, it cannot be generalized. The size of the head capsule is related to body size at the outset of the instar as Nijhout (1975) has clearly shown. Thus, under conditions of malnutrition, larvae can molt over a continuous weight range with a corresponding range of head capsule sizes. Manifestly under these conditions Dyar's rule can be used neither to predict the size at which larvae will undertake a molt nor to determine the larval instar. Consequently, Dyar's law is valid only under superior growth conditions.

Under the steady-weight regimen employed here, fourth instars exhibited an inverse relationship between instar duration and their maintenance weight. This variable dependence of molt initiation on body size may reflect different mechanisms at play; for example, the Dyarstat may be employed under conditions of *ad lib* feeding, whereas another mechanism, perhaps the mere passage of time, may accomplish under conditions of malnourishment what is normally mediated via the Dyarstat. Alternatively, the setting on the Dyarstat may be variable rather than absolute: in this case, the weight threshold for initiating a molt might be lowered slightly each day until it matches the actual weight of the malnourished larva. Whatever the mechanism, starvation clearly short-circuits the normal link between attainment of a particular size and the initiation of ecdysone-dependent development. This appeared to be a general effect of malnourishment in these studies since larvae from the third, fourth, and fifth instars, when underfed, regularly initiated development at a fraction of their typical mature weights.

In the accompanying paper (Safranek and Williams, 1984) we demonstrate that malnourished fifth-instar larvae are able to initiate metamorphosis only if they have attained a weight of at least about 3 g. This development threshold differs fundamentally from the weight thresholds for larval molting that we describe here. Thus, whereas larval molting may occur at virtually any weight in at least the late third, fourth, and early fifth instars, initiation of metamorphosis absolutely required attainment of a weight beyond the interval of 2 to 3 g. Moreover, whereas under conditions of *ad lib* feeding the initiation of larval molting requires a several-fold increase in body size within an instar, the initiation of metamorphosis in the final instar depends only on the attainment of a body size of 2–3 g regardless of the fold increase in body weight. Thus, larvae manifest abilities to gauge their size both relative to earlier points in the instar or life history as well as on an absolute scale.

Under conditions of *ad lib* feeding the initiation of molting normally occurs in strict relation to the photocycle after the attainment of an appropriate weight. And though under adverse feeding conditions molting was initiated at much lower weights, even under these conditions the initiation of molting appeared to be closely tied to the photocycle. Apparently when molting occurs under conditions of severe malnourishment it continues to be properly orchestrated and does not represent merely the breakdown of normal control mechanisms.

Assays of PTTH-activity in the brains of young fourth-instar larvae under conditions both of *ad lib* feeding and of malnutrition revealed levels of activity clearly greater than those exhibited by the diapausing pupal brain and similar to those of non-diapausing pupal brains as well as of brains from mature fourths about to initiate a molt. These findings suggest that the failure of young intermolt larvae or of malnourished larvae to initiate a molt is not due to a diapause-like condition of the brain at these periods.

In the absence of adequate data detailing the pattern of hemolymph PTTH titers in hornworm larvae, these experiments raise the possibility that our present models describing the regulation of larval molting are incomplete. Certainly the requirement for a brain is absolute, since even mature fourth-instar larvae deprived of their brains

just before molt initiation uniformly fail to undertake any subsequent development, although they may live for over two weeks (Safranek and Williams, 1980). But whether a distinct change in the PTTH output of the brain occurs at the outset of a molt as is classically envisioned is by no means certain. Certainly a significant body of observations in the literature documents that some insects can generate a molt-inducing level of ecdysone in the absence of the brain, among these the hornworm itself from the final instar through the subsequent metamorphic molts (Safranek and Williams, 1980). Manifestly PTTH-independent mechanisms for driving a molt exist, and these may well prove to be critically involved even in molts such as the hornworm's larval-larval molts where the presence of the brain is indispensable. Though some mechanism must operate near the outset of a larval molt to integrate information concerning larval size, duration of the instar, and phase of the photocyle, it is by no means clear whether the brain plays the role of maestro or of metronome.

ACKNOWLEDGMENTS

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LITERATURE CITED

BECK, S. D. 1950. Nutrition of the European corn borer *Pyrausta nubilalis* (Hbn.). II. Some effects of diet on larval growth characteristics. *Physiol. Zool.* **23:** 353–361.

BECKEL, W. E., AND W. G. FRIEND. 1964. The relation of abdominal distention and nutrition to molting in *Rhodnius prolixus* (Stahl) (Hemiptera). *Can. J. Zool.* **42:** 71–78.

BELL, R. A., AND F. G. JOACHIM. 1976. Techniques for rearing laboratory colonies of tobacco hornworms and pink bollworms. *Ann. Entomol. Soc. Am.* **69:** 365–373.

BLAKLEY, N., AND S. R. GOODNER. 1978. Size-dependent timing of metamorphosis in milkweed bugs (*Oncopeltus*) and its life history implications. *Biol. Bull.* **55:** 499–510.

BODENHEIMER, F. S. 1933. The progression factor in insect growth. *Q. Rev. Biol.* **8:** 92–95.

DYAR, H. G. 1890. The number of molts of lepidopterous larvae. *Psyche* **5:** 420–422.

JONES, D., G. JONES, AND B. D. HAMMOCK. 1981. Growth parameters associated with endocrine events in larval *Trichoplusia ni* (Hubner) and timing of these events with developmental markers. *J. Insect Physiol.* **27:** 779–788.

NIJHOUT, H. F. 1975. A threshold size for metamorphosis in the tobacco hornworm, *Manduca sexta* (L.). *Biol. Bull.* **149:** 214–225.

NIJHOUT, H. F. 1979. Stretch-induced moulting in *Oncopeltus fasciatus*. *J. Insect Physiol.* **25:** 277–281.

SAFRANEK, L., AND C. M. WILLIAMS. 1980. Studies of the prothoracicotropic hormone in the tobacco hornworm, *Manduca sexta*. *Biol. Bull.* **158:** 141–153.

SAFRANEK, L., AND C. M. WILLIAMS. 1984. Critical weights for metamorphosis in the tobacco hornworm, *Manduca sexta*. *Biol. Bull.* **167:** 555–567.

TRUMAN, J. W. 1972. Physiology of insect rhythms. I. Circadian organization of the endocrine events underlying the moulting cycle of larval tobacco hornworms. *J. Exp. Biol.* **57:** 805–820.

TRUMAN, J. W., AND L. M. RIDDIFORD. 1974. Physiology of insect rhythms. III. The temporal organization of the endocrine events underlying pupation of the tobacco hornworm. *J. Exp. Biol.* **60:** 371–382.

Reference: *Biol. Bull.* **167:** 579–589. (December, 1984)

FOOD-INDUCED SIZE-SPECIFIC MOLT SYNCHRONY OF THE SAND CRAB, *EMERITA ANALOGA* (STIMPSON)

PAUL R. SIEGEL

Department of Biological Sciences, University of California, Santa Barbara, California 93106

ABSTRACT

Synchronous molting was found in both laboratory and field populations of the intertidal, filter feeding anomuran *Emerita analoga*. Molt synchrony resulted in distinct peak and trough molting periods which were apparently independent of lunar phase and not pheromonally entrained. The intermolt period for *Emerita* was correlated with animal size.

Laboratory experiments showed that the molt cycles of a previously synchronized group of female *Emerita analoga* could be desynchronized and resynchronized by altering feeding regimes.

It is proposed that in nature the increase in phytoplankton, which characterizes the spring bloom, entrains the synchronous molting rhythm observed in the field.

Synchrony tends to be obscured in field samples composed of broad size ranges because of the size-specific character of the intermolt period. For animals which molt synchronously, estimates of growth rate based on molt frequency data extrapolated from field samples can be variable and misleading.

INTRODUCTION

Generally, molt synchrony is considered to be a consequence of selective pressures which result in either the improved adaptation of an organism to its physical environment or a reduction of molting related mortality (Reaka, 1976). Synchronous molting has been recognized in a number of marine Crustacea, and many studies have been undertaken to determine how the molt cycles of animals in a population become synchronized. In many species, physical factors such as water temperature (Carlisle, 1953; Dall, 1965; Webster, 1981), photoperiod (Aiken and Waddy, 1976; Conan, 1984), lunar phase (Braley, 1979; La Hue, 1981), and tidal cycles (Klapow, 1972; Reaka, 1976) have been implicated as synchronizers ("zeitgebers"). In others, molting cycles have been synchronized by biological factors such as pheromones (Howe, 1981), the interaction of growth and reproductive hormone systems (Scudamore, 1948; Conan, 1984), the periodic availability of food (Joose and Testerinck, 1977), or synchronous hatching (Dagg, 1976).

During studies on the role of food availability on molt synchrony it has been difficult to determine whether synchronous feeding was a cause or a result of molt cycle synchronization (Klapow, 1972). Crustacea generally do not feed during certain phases of the molting cycle (Passano, 1960); therefore, the cyclic presence or absence of food in the gut of an animal under study can be confusing.

In the present study, the molting synchrony of the sand crab, *Emerita analoga*, was investigated and the role of various physical and biological phase setters explored.

Received 5 July 1984; accepted 28 August 1984.

Present address: Department of Zoology and Marine Biology, University of Dar es Salaam, Dar es Salaam, Tanzania, Africa.

It is proposed that the observed molt synchrony was a ramification of energy partitioning and not a direct consequence of selective pressures.

Materials and Methods

General collection, measurement, and maintenance techniques

Female *Emerita analoga* were collected from the intertidal zone of Goleta Beach, 15 km west of Santa Barbara, California. Samples containing sand and sand crabs were scooped from the beach by hand or by shovel, placed in 1 mm mesh bags and rinsed in the ocean to separate the crabs from the sand. The carapace length of crabs was determined using the method of Wenner *et al.* (1974), and a narrow size class selected for each experiment. The actual carapace length range of the size class selected for each experiment depended upon the size of crabs available on the beach at the time of collection. Animals were kept in continually flowing unfiltered sea water in circular tanks. The bottoms of the tanks were covered with 5 cm of sand in which animals could burrow. The tanks were checked daily for molts by stirring up the sand, which caused the exuvia to rise to the surface of the sand. Molt frequencies percentages were based on the number of animals alive in a tank at the end of each week.

Long term molt frequency of animals in the laboratory: 1979–1980

Eight hundred female *Emerita analoga* (carapace length 10–13.5 mm) were collected in September 1979 and measured by the above methods. Three hundred of the animals were placed in each of two circular tanks (Tanks 1, 2) and two hundred in a third (Tank 3). On 26 March 1980, 23 male *E. analoga* were added to Tank 3.

On 15 August 1980, the size and molt frequency (since the time of collection) of animals in Tanks 1 and 2 were compared. A Student's *t*-test indicated that the size of the animals in the two tanks was not different ($P > 0.1$), and a Mann-Whitney U test indicated that the molt frequency of the animals was likewise not different ($P > 0.1$). The animals in the two tanks (Tanks 1 and 2) were combined, and 106 of the crabs were removed for another experiment, leaving 52 animals which continued to be monitored in this experiment. This group of females was kept apart from males to preclude egg production.

Egg production by the females in Tank 3 (the tank to which males had been added) began in the spring of 1980. Ovigerous females were identified by the presence of a large external egg mass on the pleopods. The percentage of females with egg masses was checked every two weeks.

Intermolt period for laboratory animals: 1981

In January 1981, 150 female *Emerita analoga* were collected from Goleta Beach, measured as previously described, and divided into six size classes, three size classes of large animals (with 30 animals each) and three size classes of small animals (with 20 animals each) (Table I). Two size classes, a small and a large, were placed in each of three tanks. The animals in the small size classes were marked with a small hole in the lower left margin of the carapace, and the animals in the large size classes were marked with a hole on the lower right margin of the carapace. In this way the size class from which a molted animal came could be easily recognized. Molts were collected daily and molted animals were remarked. Molting of the animals in each size class in each tank was monitored through two molting cycles. Each cycle was tested for normality with a Lilliefors' test (Conover, 1980) and the mid-point of each cycle determined graphically (Harding, 1949). The intermolt period of each size class

TABLE I

Mean carapace length, number of animals per size class, mean time at first and second molting peaks, the percentage of animals molting during each peak, and the intermolt period for the size class as determined by the time between peaks

Location	Carapace length (mm + S.D.)	Mid-point 1st peak	n	% Molt/ peak	Mid-point 2nd peak	n	% Molt/ peak	Intermolt period
Tank 1								
Large	12.2 + 0.8	5 May	21	86	17 June	17	94	43
Small	10.6 + 0.4	7 May	13	115	13 June	9	100	37
Tank 2								
Large	13.5 + 0.4	7 May	27	89	20–21 June	24	104	44.5
Small	11.0 + 0.3	5 May	15	100	13 June	14	86	39
Tank 3								
Large	12.4 + 0.8	4 May	26	62	14 June	27	100	41
Small	10.4 + 0.5	10–11 May	17	89	15 June	15	100	36.5

was determined as the time between the mid-points of the molting peaks (as in Fig. 1). The percentage of animals to molt during a peak was determined by dividing the number of molts by the number of animals alive at the end of the peak.

Field molt frequency and estimated intermolt period: 1982

Estimates of molt frequency and intermolt period of female *Emerita analoga* from the field were calculated to determine how consistent such estimates would be for a synchronously molting species. The estimates were derived using the instantaneous growth rate rationale of Fusaro (1977). From July to September 1982, I collected 13 field samples from Goleta Beach. Each sample consisted of between 51 and 209 animals with 10–12.5 mm carapace length. In all, 1623 animals were collected. After

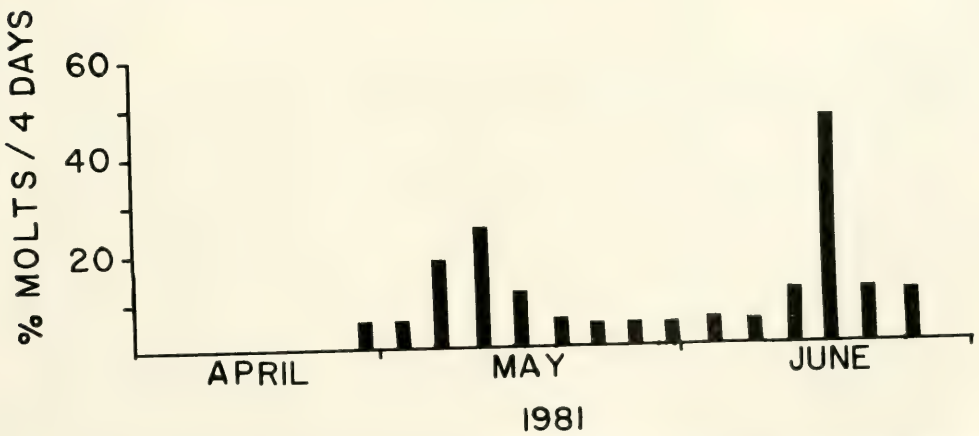

FIGURE 1. Determination of the intermolt period of a single size class of *Emerita analoga* held in the laboratory. The example shown is based on the molt frequency of the small size class from Tank 1 (Fig. 4) from April to June 1981. Molt frequency was monitored through two molt cycles. Each cycle was tested for normality and the intermolt period defined as the time between the mid-points of the two peaks.

capture, animals were kept in the laboratory in trays, maintained in flowing sea water for four days, and checked daily for molts. The intermolt period estimates (Table II) were derived from the formula $I = t/Pt$ where I was the intermolt period, t was the number of days that animals were in the laboratory (4), and Pt was the proportion of animals from the sample to molt during time t (Fusaro, 1977). For example, if 20% of the animals molted in 4 days, the estimated intermolt period would be 4/0.2 or 20 days.

Food as a synchronizer of molting: 1982–1983

On 27 September 1982, 90 female *Emerita analoga* (carapace length = 11.6 ± 0.7 mm) were collected, measured, and divided equally between three tanks (A, B, C). The tanks were supplied with a constant flow of filtered (plankton-free) sea water, thereby restricting the crabs' feeding. The animals were starved in that manner for a period of six weeks. On 9 November 1982, I began feeding the animals in Tank A each day. Two weeks later (24 Nov 1982) I began daily feeding of the animals in Tank B. Food consisted of 3.4 g of *Artemia salina* eggs hatched in 9.5 litres of sea water at 28°C for 48 hours. The animals in Tank C remained unfed for the entire experiment as a control. Tanks were checked daily for exuvia.

Feeding of the animals in both Tanks A and B was discontinued on 24 January 1983 and the animals starved for six weeks. The daily feeding of both tanks was resumed on 10 March 1983 and continued until the end of the experiment on 14 May 1983. The temporal distribution of molting events in the tanks was compared using a Smirnov test (Conover, 1980).

Results

The molt frequency of female *Emerita analoga* from January 1979 to December 1980 was characterized by significant ($P < 0.01$) peaks and troughs (Fig. 2A). Molt frequency was low during the winter and fall, resulting in broad, ill defined cycles, whereas cycles during the spring and summer were quite distinct. There were three molting cycles between April and September 1980, with approximately 100% of the animals molting during each cycle. Up to 32% of the animals molted per week during

Table II

Estimated intermolt periods based on the molt frequency of animals collected from the field and held in the laboratory for 4 days

Collection	n	% Molt/4 days	Estimated intermolt (days)
27 June	59	3.4	117.6
10 July	185	1.1	363.6
15 July	165	14.5	27.6
21 July	147	6.1	65.6
28 July	114	5.3	75.5
1 Aug	51	0.0	
7 Aug	77	9.1	44.0
13 Aug	87	8.0	50.0
18 Aug	102	1.0	400.0
25 Aug	161	14.9	26.8
31 Aug	209	4.8	83.3
8 Sept	131	2.3	173.9
22 Sept	135	0.7	571.4

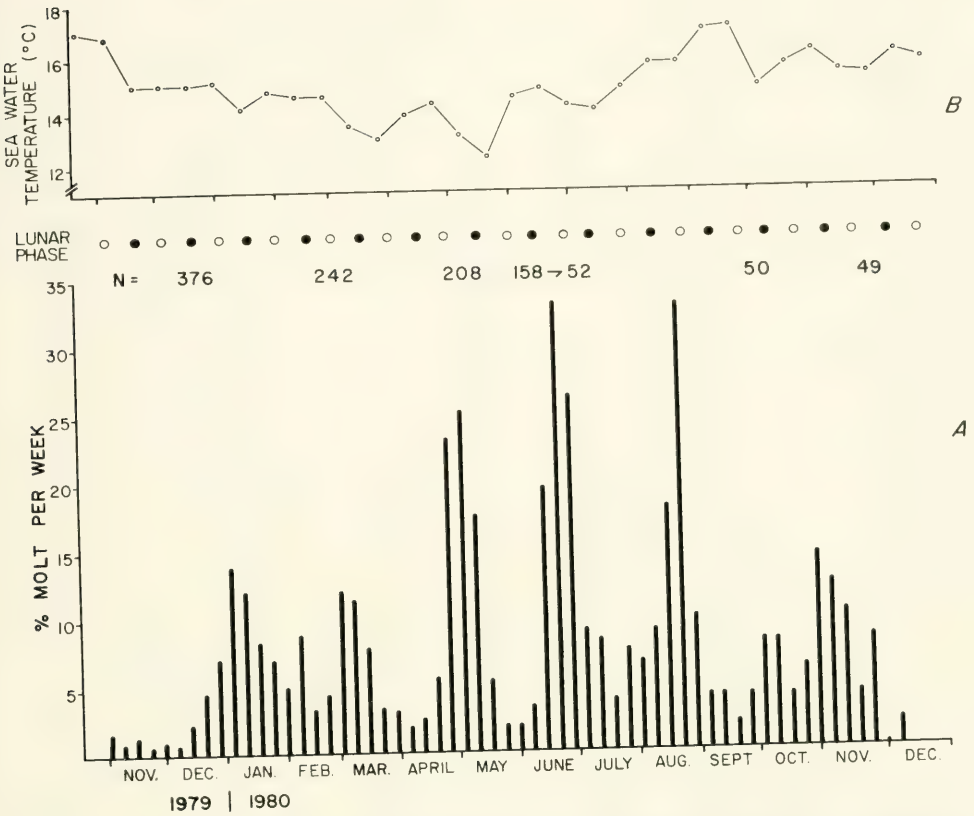

FIGURE 2. (A) Molt frequency (% molts/week) of a tank of female sand crabs, *Emerita analoga* from November 1979 to December 1980. Superscripted numbers represent the number of crabs upon which the percentages were based. The reduction in sample size in June-July was due to removal of some crabs for another experiment (see text for explanation). (B) Also shown are lunar phases (open circles = full moon, closed circles = new moon) and mean sea water temperature for the same period.

peak periods compared to 2% molting per week during trough periods. An analysis of molt frequency comparing molt frequencies during full and new moon lunar phases (Fig. 2B) to the molt frequency during first and third quarter lunar phases showed no significant difference ($P > 0.1$).

Molting cycles during the spring and summer of 1980, for females which produced eggs and equal-sized females which did not produce eggs, were characterized by three peaks: one in early May, one in late June, and one in mid-August (Fig. 3). During the same period, the percentage of ovigerous females in the tank to which males had been added increased to 95.5%, with no disruption of the synchrony phasing between the two groups.

The cyclic nature of the molt frequency was used to determine the intermolt period for six different size classes of female *Emerita analoga* (Table I, Fig. 1). A Kendall's tau test revealed that the intermolt period was positively correlated with carapace length ($P < 0.01$) (Fig. 4).

The results from the 1982 field samples, which were undertaken to detect molt synchrony in a field population, are shown in Figure 5. The percentage of females

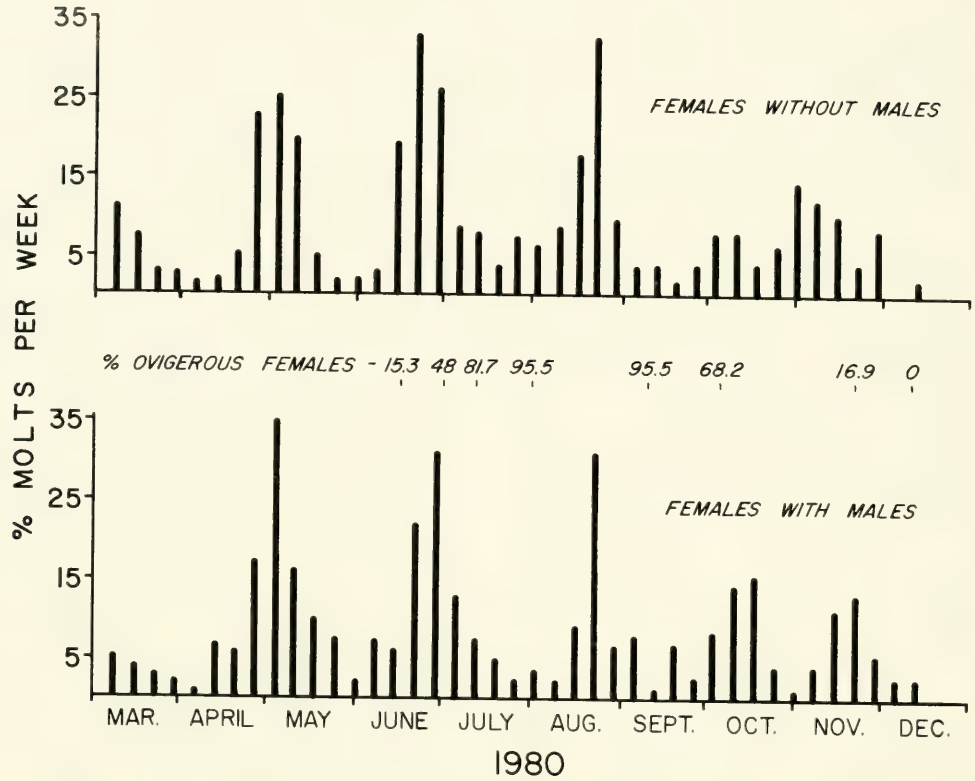

FIGURE 3. Molt frequency (% molts/week) of female sand crabs during the reproductive season of 1980. Some females were kept without males and so did not produce eggs (upper histogram) while some females kept with males (lower histogram) did produce eggs. The percentage of females to carry extruded eggs is shown between the two histograms for each date that egg production was assessed.

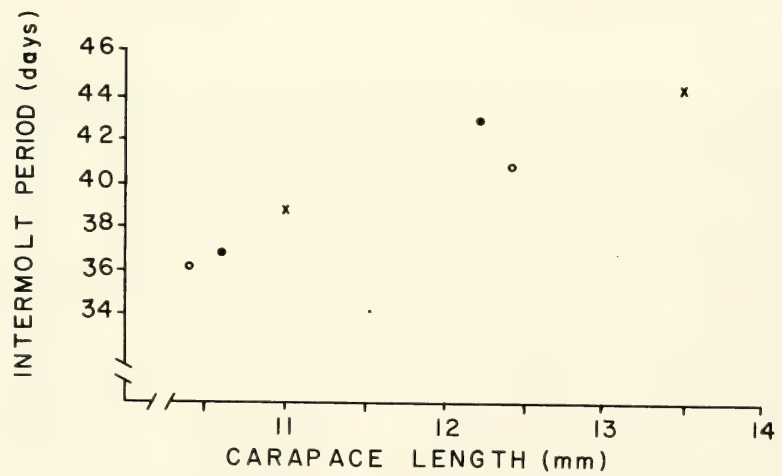

FIGURE 4. Intermolt period as a function of carapace length. Intermolt periods shown for three tanks of female *Emerita analoga* (○, ●, ×), with two size classes in each.

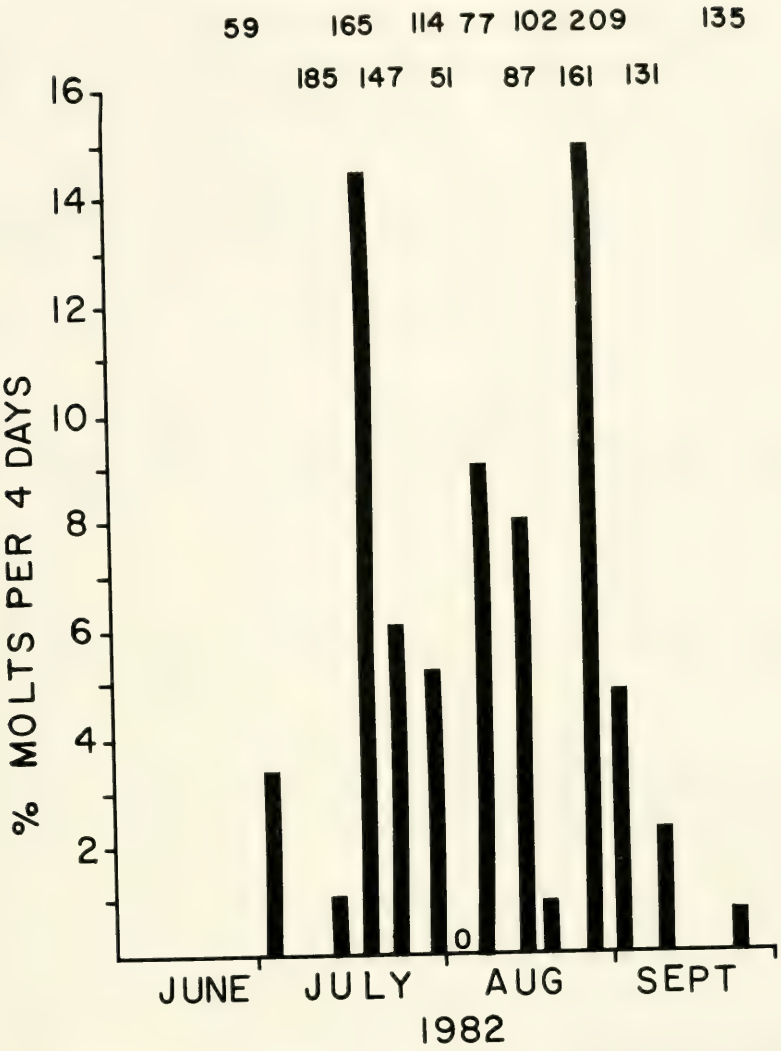

FIGURE 5. Molt frequency of female sand crabs *Emerita analoga* collected from Goleta Beach between July and September 1982. A total of 1623 animals was collected. Superscripted numbers represent the sample size upon which the % molts/4 days were based.

which molted in the laboratory over each four day holding period showed peaks and troughs which varied significantly from random ($P < 0.001$). Nearly 15% of animals collected on 15 July and 25 August molted during the four day period that each sample was in the laboratory. In contrast, less than 2% of the animals collected on 10 July, 1 August, and 18 August molted during each respective four day period. Estimates of the intermolt period (the number of days necessary for 100% of the animals to molt) for female *E. analoga* from the field ranged from less than a month to more than a year (Table II), even though the animals from each sample were the same size.

The molt cycles of animals collected from the field could be desynchronized and resynchronized using food as a desynchronizing/resynchronizing agent (Fig. 6). After

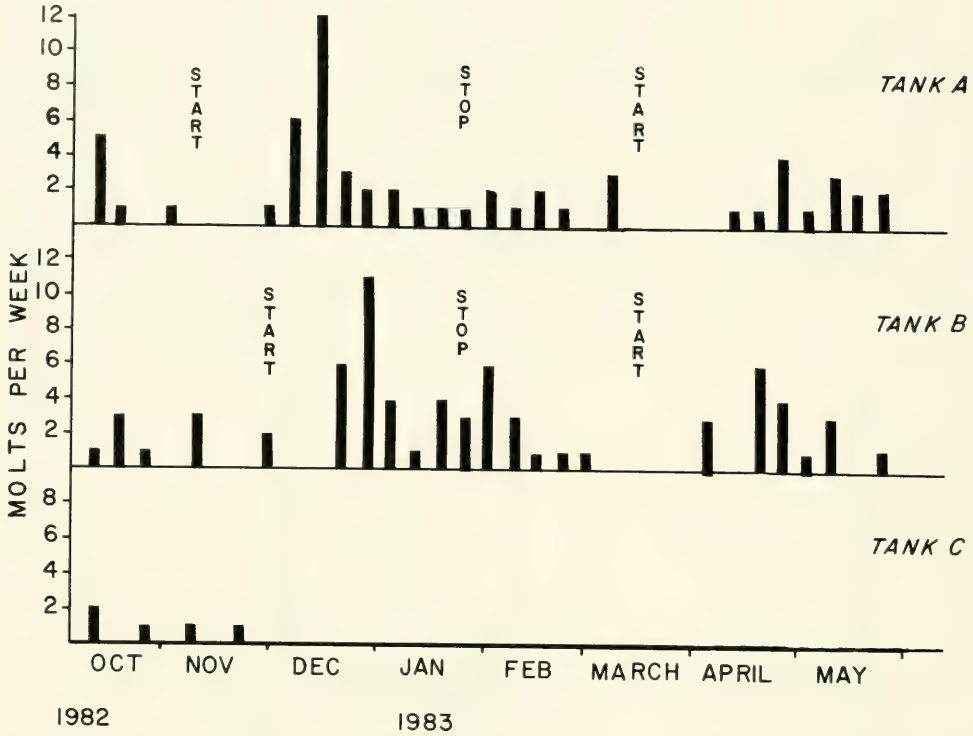

FIGURE 6. Desynchronization and resynchronization of the molt cycles of female *Emerita analoga* in response to different feeding schedules. Crabs were starved until feeding was begun (START). Daily feeding was continued until STOP and animals remained unfed until the following START. Animals in Tank C were unfed for the duration of the experiment.

the initial period of starvation and the different feeding schedules which followed, molting peaks occurred in the two tanks (Tanks A and B) two weeks apart, corresponding to the initial separation of feeding ($P < 0.05$). However, after the animals in both tanks were again starved for six weeks and feeding begun for the animals in both tanks at the same time, there was no longer a statistically significant difference in the timing of molting ($P > 0.1$).

DISCUSSION

Models of crustacean growth and production contain terms for size-specific growth rates which are derived from molt frequency and molt increment estimates. The molt frequency of field populations are often derived from the proportion of soft shelled animals caught during field samples (Conan *et al.*, 1976; Efford, 1967) or from short term laboratory experiments (Fusaro, 1978). However, unless the distribution of molting events through time is random (or constant) estimates of the molt frequency based upon such measures may greatly over or under estimate the real molt frequency. As a result of synchronous molting of female *Emerita analoga* (Fig. 5), estimates of the intermolt period varied by more than an order of magnitude (Table II).

From the preceding experiments I concluded that neither the physical factors of temperature, nor lunar phase, nor the biological factors of reproductive seasonality or pheromones played a pivotal role in the establishment or maintenance of the molt

synchrony of *Emerita analoga*. The degree to which animals were synchronized (peak amplitude) increased between April and May 1980, while the ambient sea water temperature was decreasing, and remained high while temperature both decreased and increased (Fig. 2). Periods of low molting were not associated with periods of low temperature nor did periods of high molting occur when the sea water temperature was highest. Clearly, the molting of *E. analoga* was not synchronized by the rise of sea water temperature above a physiological threshold, as has been shown for *Leander* (Carlisle, 1953), *Metapenaeus sp.* (Dall, 1965), or *Palaemon elegans* (Webster, 1981). The duration of each molting cycle was too long to be a response to semi-lunar periodicity, and statistical analysis revealed no lunar periodicity. The intermolt period for the female *Emerita analoga* represented in Figure 2 appears to reflect a bimonthly period but this was an artifact of the size class (12 mm carapace length) of the crabs (Fig. 4).

Molt synchrony was also not entrained as a consequence of egg production (Fig. 3). Females in both egg producing and non egg producing tanks showed highly synchronized molting months before the onset of egg production (June 1980). The fact that molt synchrony was uneffected by egg production was also evident from the simultaneous molting which occurred in both of those tanks ($P > 0.1$ in all cases). Synchrony was maintained between those two groups even as egg production increased, because the development time for eggs was less than the intermolt period of the females (time between peaks). Females which produced eggs molted at the same time as females which did not and produced a brood of eggs which matured and hatched before the subsequent molting peak. In this way, molting between the tanks remained synchronized without egg loss.

Molt synchrony was apparently not mediated by the action of a pheromone, as has been proposed for the synchronization of molting of *Macrobrachium rosenbergii* (Howe, 1981). In general, pheromonal induction of molt synchrony occurs when a molting animal sheds a pheromone into surrounding water, which triggers a molting response from conspecifics in the vicinity. If this has been the mechanism whereby the molt cycles of *Emerita analoga* were synchronized, then the two size classes of female crabs that were in the same tank (Fig. 4) would have tended to molt at the same time. Pheromonally induced synchrony would have resulted in simultaneous molting peaks for different size classes of animals in the same tank, and an intermolt period for the two size classes which would have been identical. Instead, the intermolt period for the six size classes shown in Figure 4 varied as a function of carapace length (Table I). A positive correlation between carapace length and intermolt period is common among decapods (Hartnoll, 1982; Mauchline, 1977) so it was not surprising to find that *E. analoga* followed that pattern.

The data presented in Figure 6 showed that the molt cycles of animals of a fixed size class could be desynchronized and resynchronized. Both desynchronization and resynchronization were induced by altered feeding schedules following a period of starvation. My interpretation of those results is that molt synchrony was a ramification of energy partitioning. The reasoning is as follows.

The energy assimilated by a non-reproductive animal is, for the most part, partitioned between growth and metabolism, and metabolic energy demands frequently vary with animal size (Vidal, 1980; Ross, 1982). If the molt frequency of the animals were determined by ration, which is common among crustacea (Hartnoll, 1984), the energetic requirement for molting would be met by all the like-sized individuals of a starved group at about the same time after food became available resulting in a pulse of molting.

Since the molt frequency of *Emerita analoga* is strongly affected by ration (Fusaro, 1977) and since sand crabs feed predominantly on phytoplankton (Osorio *et al.*, 1967), the synchronous molting pattern shown in Figure 2 can be interpreted through the theoretical framework described above. Although no phytoplankton abundance data were collected during the spring of 1979 to directly correlate the onset of the spring bloom with the onset of the spring through summer molting peaks, phytoplankton abundance off the coast of Central California is generally high during spring and summer and low during fall and winter (Eickstaedt, 1969), although occasional plankton blooms may occur at the end of the year, resulting in greater than normal sand crab growth (Siegel and Wenner, 1984). The period of highest phytoplankton abundance corresponds to the period of maximum growth of *E. analoga* while sand crab growth during the fall and winter (when phytoplankton levels are normally low) is characteristically slow (Efford, 1967; Fusaro, 1977).

During the winter (when food is normally sparse) sand crabs apparently use reserves gathered during the preceding season, and both molt frequency and degree of synchrony are low. The spring bloom, which characteristically occurs in March or April (Eickstaedt, 1969), may have provided the animals, which had been starved during the winter, with a pulse of food which was reflected in an increase in both molt frequency and degree of synchrony. The synchronous molt cycles were maintained throughout the summer since the females were the same size and had the same intermolt period, but, as food levels dwindled towards winter, both molt frequency and the degree of synchrony exhibited by the population decreased.

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LITERATURE CITED

AIKEN, D. E., AND S. L. WADDY. 1976. Controlling growth and reproduction in the American lobster. *Proc. Ann. Meet. World Maric. Soc.* **7:** 415–430.

BRALEY, R. D. 1979. Penaeid prawns in Fanga uta lagoon, Tongatapu. *Pac. Sci.* **33:** 315–321.

CARLISLE, D. B. 1953. Moulting hormones in *I* (Crustaea Decapoda). *J. Mar. Biol. Assoc. U.K.* **32:** 289–296.

CONAN, G. Y., C. MELO, AND G. YANY. 1976. Evaluation de la production d'une population littorale du crabe Hippidae *Emerita analoga* (Stimpson) par integration des parametres de croissance et de mortalite. Pp. 129–150 in *10th European Symposium on Marine Biology,* Vol. II, *Population Dynamics,* Persoone and Jaspers, eds. University Press, Wettern, Belgium.

CONAN, G. Y. in press. Periodicity and phasing of molting. In *Crustacean Issues,* Vol. 2. *Crustacean Growth,* A. M. Wenner, ed. Balkema, Netherlands.

CONOVER, W. J. 1980. *Practical Nonparametric Statistics.* John Wiley and Sons. New York. 495 pp.

DAGG, M. J. 1976. Complete carbon and nitrogen budgets for the carnivorous amphipod *Calliopius laeviusculus* (Kroyer). *Int. Rev. Ges Hydrobiol.* **61:** 297–357.

DALL, W. 1965. Studies on the physiology of a shrimp, *Metapenaeus sp.* (Crustacea: Decapoda: Penaeidae) *Aust. J. Mar. Fresh. Res.* **16:** 1–12.

EFFORD, I. E. 1967. Neoteny in the sand crab genus *Emerita* (Anomura, Hippidae). *Crustaceana* **13:** 81–93.

EICKSTAEDT, L. L. 1969. The reproductive biology of the sand crab, *Emerita analoga* (Stimpson). Ph.D. Thesis. Stanford University, Palo Alto, California.

FUSARO, C. 1977. Population structure, growth rate, and egg production of the sand crab, *Emerita analoga* (Hippidae): a comparative analysis. Ph.D. Thesis. University of California, Santa Barbara.

FUSARO, C. 1978. Growth rate of the sand crab, *Emerita analoga* (Hippidae) in two different environments. *Fish. Bull.* **76:** 369–375.

HARDING, J. P. 1949. The use of probability paper for the graphical analysis of polymodal frequency distributions. *J. Mar. Biol. Assoc. U.K.* **28:** 141–153.

HARTNOLL, R. G. 1984. Growth. Pp. 119–196 in *Biology of Crustacea,* Vol. II, *Embryology, Morphology, and Genetics,* L. G. Abele, ed. Academic Press, New York.

HOWE, N. R. 1981. Partial molting synchrony in the giant Malaysian prawn, *Macrobrachium rosenbergii:* a chemical communication hypothesis. *J. Chem. Ecol.* **7:** 487–499.

JOOSE, E. N. G., AND G. J. TESTERNIK. 1977. The role of food in the population dynamics of *Orchesella cincta* (Linne) (Collembola). *Oecologia* **29:** 189–203.

KLAPOW, L. A. 1972. Fortnightly molting and reproductive cycles in the sand beach isopod *Excirolana chiltoni. Biol. Bull.* **143:** 568–591.

LA HUE, A. K. 1981. Factors influencing molt rate and size at maturity in *Pachygrapsus crasipes* Randall (Brachyura: Grapsidae). M.A. thesis. University of California, Santa Barbara.

MAUCHLINE, J. T. 1977. Growth of shrimps, crabs and lobsters—an assessment. *J. Cons. Int. Explor. Mer.* **37:** 162–169.

OSORIO, C., N. BAHAMONDE, AND M. T. LOPEZ. 1967. El limache [*Emerita analoga* (Stimpson)] en Chile. *Boll. Mus. Nac. Hist. Nat.* **29:** 61–116.

PASSANO, L. M. 1960. Molting and its control. Pp. 473–536 in *The Physiology of Crustacea,* Vol. 1. Academic Press, New York.

REAKA, M. L. 1976. Lunar and tidal periodicity of molting and reproduction in Stomatopoda Crustacea: a selfish herd hypothesis. *Biol. Bull.* **150:** 468–490.

ROSS, R. M. 1982. Energetics of *Euphausia pacifica* 1. Effects of body carbon and nitrogen and temperature on measured and predicted production. *Mar. Biol.* **68:** 1–13.

SCUDAMORE, H. H. 1948. Factors influencing molting and the sexual cycles in the crayfish. *Biol. Bull.* **95:** 229–237.

SIEGEL, P. R., AND A. M. WENNER. 1984 (in press). Field and laboratory studies of sand crab growth. In *Crustacean Issues,* Vol. 2, *Crustacean Growth,*A. M. Wenner, ed. Balkema, Netherlands.

VIDAL, J. 1980. Physioecology of zooplankton. IV. Effects of phytoplankton concentration, temperature, and body size on the net production efficiency of *Calanus pacificus. Mar. Biol.* **56:** 203–211.

WEBSTER, S. G. 1981. Seasonal anecdysis and molting synchrony in field populations of *Palaemon elegans* (Rathke). *Estuarine Coastal Shelf Sci.* **15:** 85–94.

WENNER, A. M., C. FUSARO, AND A. OATEN. 1974. Size at onset of sexual maturity and growth rate in crustacean populations. *Can. J. Zool.* **52:** 1095–1106.

Reference: *Biol. Bull.* **167**: 590–600. (December, 1984)

HOST FEEDING REGIME AND ZOOXANTHELLAL PHOTOSYNTHESIS IN THE ANEMONE, *AIPTASIA PALLIDA* (VERRILL)

WILLIAM S. CLAYTON, JR.[1] AND HOWARD R. LASKER

Department of Biological Sciences, State University of New York at Buffalo, Buffalo, New York, 14260

ABSTRACT

Oxygen production was measured in the anemone *Aiptasia pallida* (Verrill) maintained at varying feeding regimes in the laboratory. Host feeding regime had no significant effect on: (1) zooxanthellal gross photosynthesis (GPP_{max}) expressed as $\mu g\ O_2 \cdot \mu g\ chl\ a^{-1} \cdot h^{-1}$ or $\mu g\ O_2 \cdot 10^6$ zooxanthellae$^{-1} \cdot h^{-1}$; (2) the light intensity at which $\frac{1}{2}\ GPP_{max}$ occurs (K_m); or (3) the chlorophyll *a* content of the zooxanthellae. Starvation significantly reduced GPP_{max} expressed as $\mu g\ O_2 \cdot mg$ host protein$^{-1} \cdot h^{-1}$ and zooxanthellal density within the host. Zooxanthellal translocation efficiency was measured by short term incubations of anemones in $NaH^{14}CO_3$. Host feeding regime had no effect on the percentage of ^{14}C translocated to the host.

These results suggest that, under these conditions, starvation did not alter the photosynthetic capability of the zooxanthellae but decreased total photosynthate translocated to the host by decreasing zooxanthellal density within the host.

INTRODUCTION

Much research on zooxanthellae-cnidarian symbioses has focused on metabolite exchange between the symbionts. Most of this work has considered the release of photosynthate by the zooxanthellae (Muscatine, 1967; Von Holt and Von Holt, 1968a; Muscatine and Cernichiari, 1969; Lewis and Smith, 1971; Trench, 1971a; Muscatine *et al.*, 1972; Patton and Burris, 1983) and its subsequent utilization by the host (Muscatine and Hand, 1958; Goreau and Goreau, 1960; Von Holt and Von Holt, 1968b; Muscatine and Cernichiari, 1969; Trench, 1971b, Fitt and Pardy, 1981). In addition to photosynthate translocation there is evidence for movement of organic and inorganic metabolites from host to the zooxanthellae (Cook, 1971; Trench, 1979; Carroll and Blanquet, 1982).

The significance of host metabolite transfer for the zooxanthellae has been examined indirectly by altering host feeding regime and measuring symbiont photosynthesis. In the temperate coral *Astrangia danae* (Milne Edwards & Haime) zooplankton feeding increases zooxanthellal photosynthetic rate (Jacques and Pilson, 1980; Szmant-Froelich, 1981) as well as host protein, lipid, and carbohydrate content (Szmant-Froelich and Pilson, 1980). This indicates that some aspect of host nutritional state affects zooxanthellal photosynthesis (Szmant-Froelich, 1981). Jacques and Pilson (1980) suggest that ammonia production by well fed hosts may be the stimulatory factor. However, the only direct evidence that zooxanthellal photosynthesis is affected by nitrogen is that of Taylor (1978) who has shown that ammonia and urea additions increased photosynthetic rate and excretion by zooxanthellae in *Acropora cervicornis* (Lamarck) *in vitro* and *in vivo*.

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[1] Present address: Department of Biology, Gettysburg College, Gettysburg, Pennsylvania, 17325.

Host-zooxanthellae transfer plays a major role in the nitrogen balance of the symbiosis and the presence of zooxanthellae enhances inorganic nitrogen uptake. For instance, symbiotic individuals of many species take up ammonia while aposymbionts and nonsymbionts excrete ammonia (Cates and McLaughlin, 1976; D'Elia and Webb, 1977; Muscatine and D'Elia, 1978; Muscatine *et al.*, 1979; Muscatine and Marian, 1982). Nutrient uptake rates for isolated zooxanthellae are similar to those for intact coral-zooxanthellae symbioses, further suggesting that it is the zooxanthellae that enhance nutrient uptake (D'Elia *et al.*, 1983). Symbiotic colonies of *A. danae* fed in bright light excrete more nitrogen than starved colonies but less than aposymbiotic colonies (Szmant-Froelich and Pilson, 1977). These results suggest that zooplankton feeding increases host nitrogen production and that zooxanthellae enhance nitrogen retention within the host.

The hypothesis that host metabolism may affect CO_2 availability for the zooxanthellae and the possibility that CO_2 produced by host respiration may stimulate the photosynthetic oxygen evolution of endosymbiotic algae has been investigated in several symbioses. In freshwater systems when the CO_2 concentration is low (<1.0 m*M* HCO_3^-), algal photosynthesis is higher within host cells than when isolated from the host (Reiser, 1980; Phipps and Pardy, 1982), suggesting that host produced CO_2 stimulates photosynthesis (Phipps and Pardy, 1982). However, when the inorganic carbon concentration is elevated (≥ 1.0 m*M* HCO_3^-), algal photosynthesis is not enhanced within host cells but inhibited (Cantor and Rahat, 1982). In marine systems where the CO_2 concentration is naturally high (>2.3 m*M* HCO_3^-), zooxanthellal photosynthesis is unaffected by inorganic carbon concentration (Burris *et al.*, 1983). These results suggest that host produced CO_2 does not affect zooxanthellal photosynthesis since CO_2 is abundant in most marine systems (Burris *et al.*, 1983).

In this study we examine the relationship between host zooplankton feeding and zooxanthellal photosynthetic oxygen production in the anemone *Aiptasia pallida.* Results suggest that, under laboratory conditions, zooplankton feeding increased the maximum per individual anemone photosynthetic rate by increasing zooxanthellal density within the host.

Materials and Methods

Specimen maintenance

Specimens of *Aiptasia pallida* originally derived from a single individual obtained from Carolina Biological Supply were maintained in artificial sea water (Instant Ocean) at 26 ± 1°C on a 14 h light/10 h dark photoperiod with a light intensity of 65 $\mu E \cdot m^{-2} \cdot s^{-1}$. Aposymbionts were produced by culturing anemones in a theoretical concentration of 10^{-5} *M* 3-(3,4 dichlorophenyl)-1,1 dimethyl urea (DCMU) for 4–5 weeks. Since DCMU is partially insoluble in sea water (Vandermeulen *et al.*, 1972) the actual concentration used was probably less than 10^{-5} *M*. Cultures were either starved, fed to repletion 1× per week, or 3× per week with freshly hatched (24–48 h) *Artemia salina* nauplii for 10 weeks prior to experiments. These treatments provided a large range of individual sizes for experiments. The size range (measured by oral disc diameter and host protein content) of anemones chosen for photosynthetic measurements overlapped considerably between treatments. This was done so that differences in photosynthetic rate between treatments would not be due to individual size differences resulting from the various treatments. With the exception of starved individuals, anemones were used in experiments 24 h prior to their next scheduled feeding.

Photosynthetic measurements

Oxygen flux of individual anemones was measured with a closed respirometry unit. The unit consisted of six 20-ml vials (20 mm dia.) vertically secured in a plexiglass chamber (19.0 cm × 10.5 cm × 7.0 cm) through which constant temperature water (26 ± 1°C) was pumped by a circulating water bath. Each vial contained a magnetic stirbar and was sealed with an oxygen probe (Yellow Springs Instruments, model# 5331) inside a plexiglass cylinder. Vial volume when sealed was 5.4 ml. During acclimation and between measurements vials were continuously flushed with sea water at a flow rate of 2 $ml \cdot min^{-1}$ using a multi-channel peristaltic pump. During measurements flow through the vials was discontinued. A dataplex (Cole-Palmer Dataplex 10, model# 8388) connected to the oxygen probes sequentially scanned each vial and generated a continuous chart recording of O_2 concentration for each vial.

Photosynthetic measurements of individuals from each feeding regime were conducted over several days. Individual anemones from each feeding regime were placed in vials and permitted to attach to the sidewalls and expand (30–45 min). Vials were then placed in the chamber such that the anemone oral disc was perpendicular to the path of light from the light source and preincubated in the dark for 15–20 min prior to a 10–20 min respiration measurement. Net photosynthetic measurements followed respiration measurements. A bank of four incandescent flood lights (Ken-Rad Reflector, 75 Watt, 120 V) was set at varying distances from the chamber to obtain light intensities ranging from 50–400 $\mu E \cdot m^{-2} \cdot s^{-1}$. The intensity of photosynthetically active radiation (400–700 nm) was measured behind the chamber with a Li-Cor Quantum Flux Meter (sensor# LI-190S). Net photosynthetic measurements were determined sequentially at increasing light intensities, with a 15–20 min acclimatization at each light level. An individual anemone spent <4 h in a vial and at no time did the oxygen concentration change >20% from initial values, with most experiments varying <10%. A control vial without an anemone was simultaneously monitored in all experiments to correct for the biological oxygen demand of sea water.

Assuming total respiration rate of host and symbiont in the light was equal to total respiration rate that was measured in the dark we estimated zooxanthellal gross photosynthesis (GPP) of individual anemones at each light intensity by summing the respiration and net photosynthesis measurements. Individual anemone GPP was then expressed as $\mu g\ O_2 \cdot \mu g\ chl\ a^{-1} \cdot h^{-1}$, $\mu g\ O_2 \cdot 10^6\ zooxanthellae^{-1} \cdot h^{-1}$ and $\mu g\ O_2 \cdot mg\ host\ protein^{-1} \cdot h^{-1}$ and GPP_{max} and K_m values determined using the Lineweaver-Burk estimate of the right rectangular hyperbola function (McCloskey *et al.*, 1978; Muscatine, 1980).

Chalker (1980, 1981) has advocated use of the hyperbolic tangent function for simulating light saturation curves for photosynthetic oxygen production. Chalker (1981) argues that this function yields a higher r^2 and narrower confidence intervals for the various photosynthetic parameters. Use of the hyperbolic tangent function in this study would not alter the significant differences found in GPP_{max} between treatments since these differences were discerned using values of GPP_{max} estimated from the less powerful right rectangular hyperbola function. Using values of GPP_{max} with narrower confidence intervals generated from the hyperbolic tangent function would only increase the level of significance for GPP_{max} between treatments.

Tissue processing

At the completion of each experiment, anemones were homogenized in 3 ml distilled water with a teflon pestle for 30 s. The resulting suspension was centrifuged

at 3400 rev/min for 3 min and the animal supernatant withdrawn by pipetting. The zooxanthellal pellet was then washed with 2 ml distilled water, centrifuged, and the wash added to the animal supernatant. Supernatant volume was increased to 10 ml with distilled water and replicate aliquots of this anemone fraction were withdrawn and assayed for protein content (Lowry *et al.*, 1951). The zooxanthellal pellet was resuspended in 4 ml by vortexing for 10 s and 1 ml withdrawn and frozen for future zooxanthellae counts. Lysed cells were not observed in these samples and zooxanthellal pellets isolated from other anemones and suspended in distilled water showed no lysis for >1.5 h (at 300×). Six replicate counts of each zooxanthellal sample were made using a hemacytometer. The remaining suspension was centrifuged and the zooxanthellal pellet extracted with 100% acetone for 24 h at −10°C. Solvent extinction was measured with a spectrophotometer (Gilford Instruments, model# 260 or Beckman Instruments, model# DB 1401) and chlorophyll content of the zooxanthellal pellet estimated using the equations of Jeffrey and Humphrey (1975). Tissue processing never took >30 min for any experiment.

Translocation experiments

Groups of three anemones (two symbiotic and one aposymbiotic) were incubated in separate vials with 20 ml Millepore filtered (.45 μm) sea water and allowed to attach and expand. $NaH^{14}CO_3$ was then added (final concentration = 0.25 μCi/ml) and the anemones incubated for 30 min at 26 ± 1°C and 65 $\mu E \cdot m^{-2} \cdot s^{-1}$. Following the incubation, anemones were rinsed three times with Millepore filtered sea water, homogenized, and separated as described previously. Aliquots of the animal and algal fractions were acidified and activity determined with a liquid scintillation counter (Beckman Instruments, model# LS 8000).

Counts per minute of all animal and algal samples were corrected for background, quenching, and counting efficiency and converted to disintegrations per minute. Aposymbiotic anemones in each vial served as controls for animal uptake in all experiments. Replicate aliquots of the animal fractions of all anemones were assayed for protein content and the activity of the animal fractions of symbiotic individuals were corrected for aposymbiont activity on a per unit protein basis.

Mutual contamination of zooxanthellae and host tissue

Contamination of isolated zooxanthellae by host tissue during separation was estimated by incubating aposymbiotic *A. pallida* in Millepore filtered sea water enriched with ^{14}C-glycerol (final concentration = 0.05 μCi/ml) at 26 ± 1°C for 4 h. Individuals were rinsed three times, homogenized for 30 s, and added to non-labelled zooxanthellae. The mixture was vortexed for 10 s, separated, and activity of the zooxanthellal and anemone fractions determined. Four analyses showed contamination of zooxanthellae by ^{14}C of 14.15 ± 1.27% ($\bar{x}$ ± S.E.).

Contamination of host tissue by zooxanthellae during separation was estimated by incubating isolated zooxanthellae in Millepore filtered sea water enriched with $NaH^{14}CO_3$ (final concentration = 0.45 μCi/ml) at 26 ± 1°C and 65 $\mu E \cdot m^{-2} \cdot s^{-1}$ for 5 h. Labelled zooxanthellae were isolated by centrifugation, suspended in unlabelled sea water for 18 h, and then centrifuged. The zooxanthellal pellet was rinsed twice with unlabelled sea water and combined with boiled host homogenate. Boiled homogenate was used since it does not stimulate zooxanthellal liberation of ^{14}C (Muscatine, 1967; Trench, 1971c; Muscatine *et al.*, 1972). The mixture was vortexed for 10 s, separated, and the activity of zooxanthellal and anemone fractions determined. Four analyses showed contamination of host tissue by ^{14}C of 3.69 ± 0.47% ($\bar{x}$ ± S.E.).

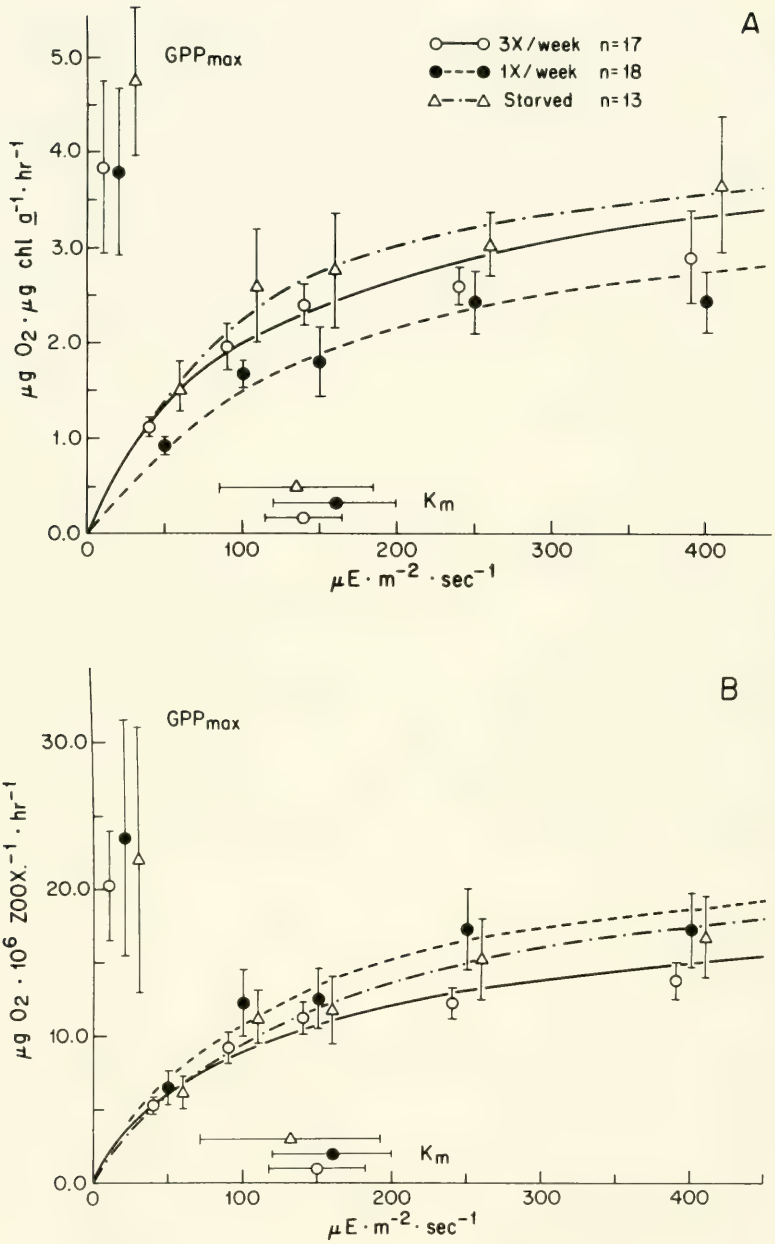

FIGURE 1. Ideal curves of the photosynthesis-irradiance relationship for zooxanthellae from *A. pallida* maintained at varying feeding regimes. GPP_{max} and K_m were calculated from the Michaelis-Menton equation. Bars for GPP_{max} and K_m denote 95% confidence limits; bars for GPP at each light intensity denote standard errors. Points at each light intensity denote the $\bar{x}$ of n measurements and are offset for clarity. A. GPP expressed as $\mu g\ O_2 \cdot \mu g\ chl\ a^{-1} \cdot h^{-1}$. B. GPP expressed as $\mu g\ O_2 \cdot 10^6\ zoox.^{-1} \cdot h^{-1}$. C. GPP expressed as $\mu g\ O_2 \cdot mg\ host\ protein^{-1} \cdot h^{-1}$.

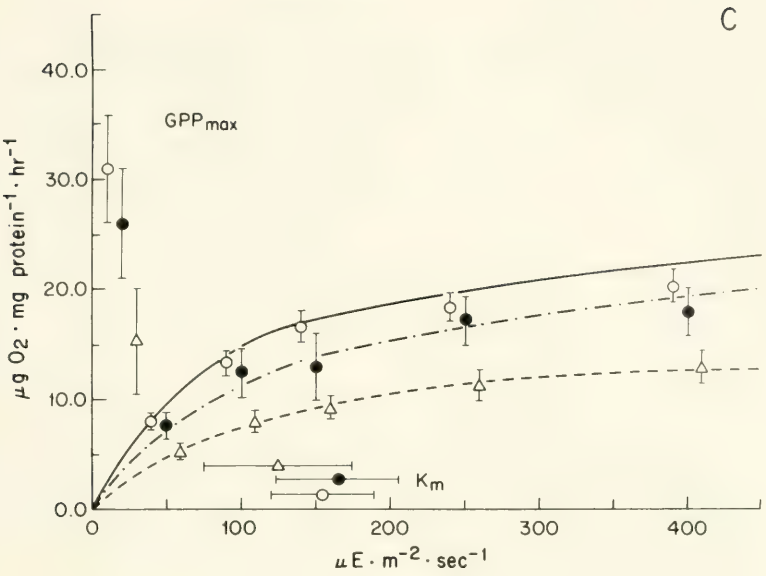

FIGURE 1. (*Continued*)

Activity of zooxanthellal and anemone fractions in the translocation experiments was corrected for mutual contamination as estimated in these experiments.

RESULTS

Zooxanthellal photosynthesis

Results of the photosynthetic measurements are presented in Figure 1a–c. All GPP_{max} and K_m values were analyzed using a two-level nested analysis of variance (ANOVA) for unequal sample sizes (Sokal and Rohlf, 1969) that tested for effects among days within a feeding regime and among feeding regimes. There was no significant difference in GPP_{max} expressed as $\mu g\ O_2 \cdot \mu g$ chl $a^{-1} \cdot h^{-1}$ ($GPP_{max}/\mu g$ chl *a*, Fig. 1a) among days ($P > .10$; df = 11, 34; F = 1.72) or feeding regimes ($P > .50$; df = 2, 11; F = 0.69). Similarly, K_m for $GPP_{max}/\mu g$ chl *a* showed no significant day ($P > .25$; df = 11, 34; F = 1.22) or feeding regime effects ($P > .75$; df = 2, 11; F = 0.29). Zooxanthellal GPP_{max} expressed as $\mu g\ O_2 \cdot 10^6$ zooxanthellae$^{-1} \cdot h^{-1}$ ($GPP_{max}/10^6$ zoox., Fig. 1b) showed a significant day effect ($P < .001$; df = 11, 34; F = 3.95), suggesting some degree of daily variation in zooxanthellal photosynthetic rate. There was no feeding regime effect for $GPP_{max}/10^6$ zoox. ($P > .50$; df = 2, 11; F = 0.48). K_m for $GPP_{max}/10^6$ zoox. showed no significant feeding regime ($P > .50$; df = 2, 11; F = 0.33) or day effects ($P > .05$; df = 11, 34; F = 1.89). GPP_{max} expressed as $\mu g\ O_2 \cdot$ mg host protein$^{-1} \cdot h^{-1}$ (GPP_{max}/mg protein, Fig. 1c) showed significant day ($P < .05$; df = 11, 34; F = 2.96) and feeding regime effects ($P < .05$; df = 2, 11; F = 4.13). K_m for GPP_{max}/mg protein showed no significant day ($P > .25$; df = 11, 34; F = 1.19) or feeding regime effects ($P > .50$; df = 2, 11; F = 0.35).

Respiration

Total individual respiration was expressed as $\mu g\ O_2 \cdot$ mg host protein$^{-1} \cdot h^{-1}$. Results are presented in Table I and were analyzed using a two-level nested ANOVA as

TABLE I

Respiration rate ($\bar{x} \pm$ 95% confidence limits) of anemones from varying feeding regimes expressed as $\mu g\ O_2 \cdot mg\ host\ protein^{-1} \cdot h^{-1}$

3×/week	1×/week	0×/week
10.26 ± 1.16	7.73 ± 1.17	3.28 ± 2.13

described for analysis of the photosynthetic measurements. There was a significant difference in respiration rate among feeding regimes ($P < .001$; df = 2, 11; F = 28.04) but no significant interaction effect ($P > .50$; df = 11,34; F = 0.82).

Zooxanthellal density, chlorophyll content, and percent translocation

All results are presented in Table II and each parameter was analyzed using a two-level nested ANOVA as described previously. Starvation reduced zooxanthellal density within the host. There was a significant difference in 10^6 zoox. · mg protein^{-1} among feeding regimes ($P < .01$; df = 2, 11; F = 8.42) but no significant difference among days ($P > .10$; df = 11, 34; F = 1.69). Feeding regime had no effect on the chlorophyll *a* content of zooxanthellae ($P > .50$; df = 2, 34; F = 0.55). However, there was a significant day effect for μg chl $a \cdot 10^6$ zoox.$^{-1}$ ($P < .001$; df = 11, 34; F = 5.05), suggesting some variation in zooxanthellal chlorophyll *a* content. Percent translocation from zooxanthellae to host was also unaffected by feeding regime. Analysis of the arcsine transformed data showed no significant day ($P > .75$; df = 9, 12; F = 0.99) or feeding regime effects ($P > .10$; df = 2, 9; F = 4.09).

DISCUSSION

Altering host feeding regime provides a tool for studying possible host effects on the zooxanthellae. Host zooplankton feeding may affect zooxanthellal photosynthetic oxygen production in two fundamental ways, via changes in the number of zooxanthellae within the host or via changes in individual zooxanthellal photosynthesis. Changes in individual zooxanthellal photosynthesis can be further compartmentalized to changes in the size or number of photosynthetic units (PSU's) within the algae. The possibilities and their predicted consequences are presented in Table III and discussed below.

Although changes in PSU size and number were not directly measured these changes should produce definable effects. Thus we can infer certain changes if the observed results are consistent with theoretical predictions. However, the predictions of this response (Table III) were not observed in the data. Feeding regime had no effect on GPP_{max}/chl *a* (Fig. 1a), K_m for $GPP_{max}/10^6$ zoox. (Fig. 1b), K_m for GPP_{max}/

TABLE II

Zooxanthellal chlorophyll a content, zooxanthellal density, and percent translocation to the host by zooxanthellae from Aiptasia pallida *maintained at varying feeding regimes ($\bar{x} \pm$ S.E.)*

	3×/week	1×/week	Starved
μg chl $a \cdot 10^6$ zoox.$^{-1}$	5.31 ± 0.73	5.99 ± 0.62	5.02 ± 0.76
10^6 zoox. · mg protein^{-1}	1.56 ± 0.10	1.24 ± 0.09	0.75 ± 0.09
% Translocation	42.73 ± 2.01	39.93 ± 1.37	45.67 ± 1.22

TABLE III

Possible effects of host zooplankton feeding on zooxanthellal photosynthetic oxygen production

Units of measure	Increasing PSU size	Increasing PSU #	Increasing zooxanthellae #
$\mu g\ O_2 \cdot \mu g\ chl\ a^{-1} \cdot h^{-1}$			
GPP_{max}	decrease*	no change*	no change
K_m	no change*	no change*	no change
$\mu g\ O_2 \cdot 10^6\ zoox.^{-1} \cdot h^{-1}$			
GPP_{max}	no change*	increase*	no change
K_m	decrease*	no change*	no change
$\mu g\ O_2 \cdot mg\ protein^{-1} \cdot h^{-1}$			
GPP_{max}	no change	increase	increase
K_m	decrease	no change	no change
$\mu g\ chl\ a \cdot 10^6\ zoox.^{-1}$	increase*	increase*	no change
$10^6\ zoox. \cdot mg\ protein^{-1}$	no change	no change	increase

* Adapted from Prezelin and Sweeney (1979).

mg protein (Fig. 1c), or μg chl $a \cdot 10^6$ zoox.$^{-1}$ (Table II). These results indicate that host zooplankton feeding did not affect zooxanthellal photosynthesis via changes in PSU size.

A second possibility for changes in individual zooxanthellal photosynthesis is an increase in PSU number. Although GPP_{max}/mg protein increased as predicted (Fig. 1c), there was no increase in $GPP_{max}/10^6$ zoox. (Fig. 1c) or μg chl $a \cdot 10^6$ zoox.$^{-1}$ (Table II). Thus the increased GPP_{max}/mg protein in fed individuals was apparently not due to increasing PSU number within the zooxanthellae.

The final possibility is that zooxanthellal density changed within the host. Although this capability is not evident as a depth related photoadaptive response (Drew, 1972; Lasker, 1977; Dustan, 1982), zooxanthellal density is not static and can be altered by environmental factors (Yonge and Nicholls, 1931a, b; Yonge *et al.*, 1932; Goreau, 1964; Jaap, 1979; Kevin and Hudson, 1979; Fankboner and Reid, 1981). Effects of host starvation on zooxanthellal density appear related to the length of starvation and are somewhat species specific. For instance, prolonged starvation reduces zooxanthellal density in some species (Yonge and Nicholls, 1931b; Smith, 1939; Taylor, 1969; Reimer, 1971) but not in others (Kevin and Hudson, 1979). Two weeks starvation reduces zooxanthellal density in *Astrangia danae* (Szmant-Froelich, 1981) but not in the anemone *Anthopleura elegantissima* Brandt (Fitt *et al.*, 1982). In addition, Franzisket (1970) showed that colonies of four coral species deprived of zooplankton in the light retained zooxanthellae and continued to grow at the same rate as when zooplankton were available. In the experiments reported here, 10 weeks starvation significantly reduced GPP_{max}/mg protein (Fig. 1c), apparently by decreasing zooxanthellal density (Table II). Host alteration of either zooxanthellal growth rate or retention of zooxanthellae associated with starvation may have altered zooxanthellal density.

The effect of host feeding regime on translocation efficiency may also affect the nutrient balance of zooxanthellae and their hosts. For instance, Szmant-Froelich (1981) found that starved *A. danae* colonies fixed less ^{14}C in photosynthesis but "compensated" by translocating more to the host. In this study, percent translocation at 26 ± 1°C was 40–45% regardless of host feeding regime (Table II). This appears

much less than the 63% translocation at 27°C recently reported for *A. pallida* (Clark and Jensen, 1982).

In this study, host feeding regime altered zooxanthellal density (Table II) but had no apparent effect on the zooxanthellal photosynthetic apparatus (Fig. 1a, b; Table II), and no effect on percent translocation by the zooxanthellae (Table II). This suggests that the significantly greater GPP_{max}/mg protein observed in fed individuals (Fig. 1c) was due to higher zooxanthellal densities (Table II) and resulted in fed individual receiving more photosynthate from their zooxanthellae population than starved individuals.

The significance of this increase in available photosynthate for fed individuals compared to starved individuals cannot be readily assessed since any increase in available photosynthate for the host due to increased feeding frequency may be offset by a concomitant increase in host respiration rate. In these experiments total respiration (of animal and algae) of individuals fed 3× per week was approximately 3× that of starved individuals after 10 weeks (Table I). A similar effect of feeding frequency on anemone respiration rate has been reported for other species (Svoboda and Porrman, 1980; Fitt and Pardy, 1981; Fitt *et al.*, 1982). Although feeding regime effects on total respiration rate are not easily partitioned between host and symbionts (*c.f.*, Muscatine and Porter, 1977; Muscatine, 1980; Muscatine *et al.*, 1981), it appears that anemone respiration rate is the major component since symbionts and aposymbionts have similar respiration rates (Pardy and White, 1977; Fitt and Pardy, 1981). In contrast with total respiration, GPP_{max}/mg protein of individuals fed 3× per week was approximately 1.5× that of starved individuals (Fig. 1c). These results suggest that the increased respiration rate associated with feeding was greater than the increased GPP_{max}/mg protein. Regardless of host utilization, however, it appears that fed anemones received more photosynthate from their zooxanthellae than did starved individuals.

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LITERATURE CITED

BURRIS, J. E., J. W. PORTER, AND W. A. LAING. 1983. Effects of carbon dioxide concentration on coral photosynthesis. *Mar. Biol.* **75:** 113–116.

CANTOR, M. H., AND M. RAHAT. 1982. Regulation of respiration and photosynthesis in *Hydra viridis* and in its separate cosymbionts: Effect of nutrients. *Physiol. Zool.* **55:** 281–288.

CARROLL, S., AND R. S. BLANQUET. 1982. Transport of amino acids from the cnidarian *Cassiopea* to its endosymbiotic zooxanthellae. *Am. Zool.* **22:** 872.

CATES, N., AND J. J. A. MCLAUGHLIN. 1976. Differences of ammonia metabolism in symbiotic and aposymbiotic *Condylactus* and *Cassiopea* spp. *J. Exp. Mar. Biol. Ecol.* **21:** 1–5.

CHALKER, B. E. 1980. Modelling light saturation curves for photosynthesis: An exponential function. *J. Theor. Biol.* **84:** 205–215.

CHALKER, B. E. 1981. Simulating light-saturation curves for photosynthesis and calcification by reef building corals. *Mar. Biol.* **63:** 135–141.

CLARK, K. B., AND K. R. JENSEN. 1982. Effects of temperature on carbon fixation and carbon budget partitioning in the zooxanthellal symbiosis of *Aiptasia pallida*. *J. Exp. Mar. Biol. Ecol.* **64:** 215–230.

COOK, C. B. 1971. Transfer of ^{35}S-labelled material from food ingested by *Aiptasia* sp. to its endosymbiotic

zooxanthellae. Pp. 218–224 in *Experimental Coelenterate Biology*. H. M. Lenhoff, L. Muscatine, and L. V. Davis, eds. Univ. of Hawaii Press, Honolulu.

D'Elia, C. F., and K. L. Webb. 1977. The dissolved nitrogen flux of reef corals. *Proc. Third Int. Coral Reef Symp.* **1:** 326–330.

D'Elia, C. F., S. L. Domotor, and K. L. Webb. 1983. Nutrient uptake kinetics of freshly isolated zooxanthellae. *Mar. Biol.* **75:** 157–167.

Drew, E. A. 1972. The biology and physiology of alga-invertebrate symbioses. II. The density of symbiotic algal cells in a number of hermatypic corals and alcyonarians from various depths. *J. Exp. Mar. Biol. Ecol.* **9:** 71–75.

Dustan, P. 1982. Depth dependent photoadaptation by zooxanthellae of the reef coral *Montastrea annularis*. *Mar. Biol.* **68:** 253–264.

Fankboner, P. V., and R. G. B. Reid. 1981. Mass expulsion of zooxanthellae by heat stressed reef corals: A source of food for giant clams? *Experentia* **37:** 251–252.

Fitt, W. K., and R. L. Pardy. 1981. Effects of starvation, and light and dark on the energy metabolism of symbiotic and aposymbiotic sea anemones, *Anthopleura elegantissima*. *Mar. Biol.* **61:** 199–205.

Fitt, W. K., R. L. Pardy, and M. M. Littler. 1982. Photosynthesis, respiration, and contribution to community productivity of the symbiotic sea anemone *Anthopleura elegantissima*. *J. Exp. Mar. Biol. Ecol.* **61:** 213–232.

Franzisket, L. 1970. The atrophy of hermatypic reef corals maintained in darkness and their subsequent regeneration in light. *Int. Revue Ges. Hydrobiol. Hydrogr.* **55:** 1–12.

Goreau, T. F. 1964. Mass expulsion of zooxanthellae from Jamaican reef communities after Hurricane Flora. *Science* **145:** 383–386.

Goreau, T. F., and N. I. Goreau. 1959. Distribution of labelled carbon in reef-building corals with and without zooxanthellae. *Science* **131:** 668–669.

Jaap, W. C. 1979. Observations on zooxanthellae expulsion at Middle Sambo reef, Florida Keys. *Bull. Mar. Sci.* **29:** 414–422.

Jacques, T. G., and M. E. Q. Pilson. 1980. Experimental ecology of the temperate scleractinian coral *Astrangia danae*. I. Partition of respiration, photosynthesis and calcification between host and symbionts. *Mar. Biol.* **60:** 167–178.

Jeffrey, S. W., and G. F. Humphrey. 1975. New spectrophotometric equations for determining chlorophylls *a*, *b* and *c* in higher plants, algae and natural phytoplankton. *Biochem. Physiol. Pflanz.* **167:** 191–194.

Kevin, K. M., and R. C. L. Hudson. 1979. The role of zooxanthellae in the hermatypic coral *Plesiastrea urvillei* from cold waters. *J. Exp. Mar. Biol. Ecol.* **36:** 157–170.

Lasker, H. R. 1977. Patterns of zooxanthellae distribution and polyp expansion in the reef coral *Montastrea cavernosa*. *Proc. Third Int. Coral Reef Symp.* **1:** 607–613.

Lewis, D. H., and D. C. Smith. 1971. The autotrophic nutrition of symbiotic marine coelenterates with special reference to hermatypic corals. I. Movement of photosynthetic products between symbionts. *Proc. R. Soc. Lond. B.* **178:** 111–129.

Lowry, O. H., H. J. Rosenborough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.

McCloskey, L. R., D. S. Wethey, and J. W. Porter. 1978. Measurement and interpretation of photosynthesis and respiration in reef corals. *Monogr. Oceanogr. Methodol.* **5:** 379–396.

Muscatine, L. 1967. Glycerol excretion by symbiotic algae from corals and *Tridacna* and its control by the host. *Science* **156:** 516–519.

Muscatine, L. 1980. Productivity of zooxanthellae. Pp. 381–402 in *Primary Productivity in the Sea*, P. G. Falkowski, ed. Plenum Press, New York.

Muscatine, L., and E. Cernichiari. 1969. Assimilation of photosynthetic products of zooxanthellae by a reef coral. *Biol. Bull.* **137:** 506–523.

Muscatine, L., and C. F. D'Elia. 1978. The uptake, retention, and release of ammonium by reef corals. *Limnol. Oceanogr.* **23:** 725–734.

Muscatine, L., and C. Hand. 1958. Direct evidence for the transfer of materials from symbiotic algae to the tissues of a coelenterate. *Proc. Natl. Acad. Sci.* **44:** 1259–1263.

Muscatine, L., and R. E. Marian. 1982. Dissolved inorganic nitrogen flux in symbiotic and nonsymbiotic medusae. *Limnol. Oceanogr.* **27:** 910–917.

Muscatine, L., and J. W. Porter. 1977. Reef corals: Mutualistic symbioses adapted to nutrient poor environments. *Bioscience* **27:** 454–460.

Muscatine, L., H. Masuda, and R. Burnap. 1979. Ammonium uptake by symbiotic and aposymbiotic reef corals. *Bull. Mar. Sci.* **29:** 554–557.

Muscatine, L., L. R. McCloskey, and R. E. Marian. 1981. Estimating the daily contribution of carbon from zooxanthellae to coral animal respiration. *Limnol. Oceanogr.* **26:** 601–611.

MUSCATINE, L., R. R. POOL, AND E. CERNICHIARI. 1972. Some factors influencing selective release of soluble organic material from reef corals. *Mar. Biol.* **13:** 298–308.

PATTON, J. S., AND J. E. BURRIS. 1983. Lipid synthesis and extrusion by freshly isolated zooxanthellae. *Mar. Biol.* **75:** 131–136.

PHIPPS, D. W., AND R. L. PARDY. 1982. Host enhancement of symbiont photosynthesis in the *Hydra*-algae symbiosis. *Biol. Bull.* **162:** 83–94.

PREZELIN, B. B., AND B. M. SWEENEY. 1979. Photoadaptation of photosynthesis in two bloom-forming dinoflagellates. Pp. 101–106 in *Toxic Dinoflagellate Blooms,* D. L. Taylor and H. H. Selinger, eds. Elsevier/North Holland Biomedical Press, New York.

REISSER, W. 1980. The metabolic interactions between *Paramecium bursaria* and *Chlorella* in the *Paramecium bursaria* symbiosis. *Arch. Microbiol.* **125:** 291–293.

SMITH, H. G. 1939. The significance of the relationship between actinians and zooxanthellae. *J. Exp. Biol.* **16:** 334–345.

SOKAL, R. R., AND F. J. ROHLF. 1969. *Biometry: The Principles and Practice of Statistics in Biological Research.* W. H. Freeman and Company, San Francisco. 776 pp.

SVOBODA, A., AND T. PORRMAN. 1980. Oxygen production and uptake by symbiotic *Aiptasia diaphana* adapted to different light intensities. Pp. 87–99 in *Nutrition in the Lower Metazoa,* D. C. Smith and Y. Tiffon eds. Pergamon Press, Oxford.

SZMANT-FROELICH, A. 1981. Coral nutrition: Comparison of the fate of ^{14}C from ingested labelled brine shrimp and from the uptake of $NaH^{14}CO_3$ by its zooxanthellae. *J. Exp. Mar. Biol. Ecol.* **55:** 133–144.

SZMANT-FROELICH, A., AND M. E. Q. PILSON. 1977. Nitrogen excretion by colonies of the temperate coral *Astrangia danae* with and without zooxanthellae. *Proc. Third Int. Coral Reef Symp.* **1:** 417–423.

SZMANT-FROELICH, A., AND M. E. Q. PILSON. 1980. The effects of feeding frequency and symbiosis with zooxanthellae on the biochemical composition of *Astrangia danae. J. Exp. Mar. Biol. Ecol.* **48:** 85–97.

TAYLOR, D. L. 1969. The nutritional relationship of *Anemonia sulcata* and its dinoflagellate symbiont. *J. Cell Sci.* **4:** 751–762.

TAYLOR, D. L. 1978. Nutrition of algal-invertebrate symbiosis. II. Effects of exogenous nitrogen sources on growth, photosynthesis and the rate of excretion by algal symbionts *in vivo* and *in vitro. Proc. R. Soc. Lond. B.* **201:** 401–412.

TRENCH, R. K. 1971a. The physiology and biochemistry of zooxanthellae symbiotic with marine coelenterates. II. Liberation of fixed ^{14}C by zooxanthellae *in vitro. Proc. R. Soc. Lond. B.* **177:** 237–250.

TRENCH, R. K. 1971b. The physiology and biochemistry of zooxanthellae symbiotic with marine coelenterates. I. The assimilation of photosynthetic products by two marine coelenterates. *Proc. R. Soc. Lond. B.* **177:** 225–235.

TRENCH, R. K. 1971c. The physiology and biochemistry of zooxanthellae symbiotic with marine coelenterates. III. The effect of homogenates of host tissue on the excretion of photosynthetic products *in vitro* by zooxanthellae from two marine coelenterates. *Proc. R. Soc. Lond. B.* **177:** 251–264.

TRENCH, R. K. 1979. The cell biology of plant-animal symbiosis. *Ann. Rev. Plant Physiol.* **30:** 485–531.

VANDERMEULEN, J. H., N. D. DAVIS, AND L. MUSCATINE. 1972. The effect of inhibitors of photosynthesis on zooxanthellae in corals and other marine coelenterates. *Mar. Biol.* **16:** 185–191.

VON HOLT, C., AND M. VON HOLT. 1968a. The secretion of organic compounds by zooxanthellae isolated from various types of *Zoanthus. Comp. Biochem. Physiol.* **24:** 83–92.

VON HOLT, C., AND M. VON HOLT. 1968b. Transfer of photosynthetic products from zooxanthellae to coelenterate hosts. *Comp. Biochem. Physiol.* **24:** 73–82.

YONGE, G. M., AND A. G. NICHOLLS. 1931a. Studies on the physiology of corals. IV. The structure, distribution and physiology of the zooxanthellae. *Sci. Rep. Great Barrier Reef Exped.* **1:** 135–176.

YONGE, G. M., AND A. G. NICHOLLS. 1931b. Studies on the physiology of corals. V. The effect of starvation in light and darkness on the relationship between corals and zooxanthellae. *Sci. Rep. Great Barrier Reef Exped.* **1:** 177–211.

YONGE, G. M., M. J. YONGE, AND A. G. NICHOLLS. 1932. Studies on the physiology of corals. VI. The relationship between respiration in corals and the production of oxygen by their zooxanthellae. *Sci. Rep. Great Barrier Reef Exped.* **1:** 215–251.

Reference: *Biol. Bull.* **167**: 601–612. (December, 1984)

PHOTOSYNTHETIC PRODUCTION BY THE CORAL REEF ANEMONE, *LEBRUNIA CORALLIGENS* WILSON, AND BEHAVIORAL CORRELATES OF TWO NUTRITIONAL STRATEGIES

JOHN B. LEWIS

Institute of Oceanography, McGill University, Montreal, Quebec, Canada H3A 2B2

Abstract

The coral reef anemone *Lebrunia coralligens* Wilson bears, in addition to its lanceolate, feeding tentacles, lobate pseudotentacles which are shown to be photosynthetic organs. Anemones exposed to light demonstrate a net oxygen production and, when incubated in $NaH^{14}CO_3$ in the light, incorporate ^{14}C into the zooxanthellae and animal tissue.

Diurnal rhythms of pseudotentacle expansion and contraction are under the control of ambient light but are modified by the animal's nutritional status. Unfed animals utilize their lobate pseudotentacles to obtain the maximum nutritional uptake by autotrophy while fed animals reduce their autotrophic intake. However, lanceolate tentacle expansion is primarily a feeding response and is augmented by a higher nutritional state. It is concluded that anemone behavior is an expression of the two nutritional strategies.

Introduction

Lebrunia coralligens Wilson is a small cryptic anthozoan which occupies cavities in dead and living coral rock (Wilson, 1890; Gladfelter, 1975). In addition to its lanceolate feeding tentacles, *L. coralligens* bears large, lobate, auxilliary extensions of the column termed pseudotentacles (Hyman, 1940). According to Gladfelter (1975) and Sebens and De Riemer (1977), the lanceolate tentacles are expanded during the night, whereas the lobate pseudotentacles are expanded during the day; the latter contain 20 to 30 times more zooxanthellae than do the true tentacles. These diurnal cycles of expansion and contraction, coupled with the presence of a large number of zooxanthellae in the pseudotentacles, have led the latter authors to conclude that *L. coralligens* is capable of both autotrophic and heterotrophic modes of nutrition.

The purpose of the present study was to confirm experimentally that the lobate pseudotentacles of *L. coralligens* are specialized photosynthetic organs, to measure their rate of production, and to determine how the two nutritional strategies may be integrated with polyp behavior.

Material and Methods

Specimens of *Lebrunia coralligens* were obtained from within rock surfaces on the fringing reefs at Barbados, West Indies (Lewis, 1960). Broken rock fragments were carried to the laboratory in sea water and the anemones carefully removed and transferred to clean glass dishes in which the water was changed twice daily. Living specimens were maintained in the laboratory for as long as two months in this manner.

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Specimens used for histological study were first incubated for 12 h in light or darkness in 350 ml of sea water to which approximately 20 μc of $NaH^{14}CO_3$ had been added, preserved in Bouin's fixative, and transferred to 70% ethanol. After imbedding in paraffin blocks, samples were sectioned at eight microns, mounted on slides smeared with albumin, and stained with hematoxylin. Slides prepared for tissue radiography were dipped in Kodak nuclear track emulsion (type N_2B_2, 1:1 emulsion:water mixture) and exposed for 10 days at 5°C. Exposed slides were developed in Kodak D-19 developer, fixed in Kodak rapid fixer, and post stained with Eosin.

To measure respiration and photosynthetic activity, anemones were incubated in darkened and light glass, snap cap vials (60 ml) for two hours (between 1100 and 1300 h). This midday period was reported to be the time of maximum lobate tentacle expansion by Gladfelter (1975) and was probably within the period of maximum photosynthesis (Fricke and Vareschi, 1982). Control vials contained no anemones. Because of the small volume of the vials, changes in oxygen concentration in the vials were determined by the following modification of the Winkler method. Twenty ml of water were withdrawn from the incubation vessel into a glass syringe. The Winkler chemicals (0.1 ml manganous sulphate, 0.1 ml alkaline iodate, and 0.2 ml sulfuric acid) were dispensed into the sampling syringe and the sample was titrated against 0.01 *N* sodium thiosulphate with a microburet. To test for accuracy, the method was compared with an unmodified Winkler technique (Strickland and Parsons, 1965). Results differed by <5%.

Observations on diurnal rhythms of tentacle expansion were conducted over periods of seven days by arrangement of six groups of twenty individuals each as follows; (a) anemones (fed on the first and fourth days) exposed to a light/dark cycle of 12 h light from 0600 to 1800, and 12 h dark from 1800 to 0600 h; (b) anemones (fed on the first and fourth days) exposed to a reverse light/dark cycle of 12 h dark from 0600 to 1800 h, and 12 h light from 1800 to 0600 h; (c) anemones exposed to continuous light with daily feeding; (d) anemones exposed to continuous light with no feeding; (e) anemones exposed to continuous darkness with daily feeding; and (f) anemones exposed to continuous darkness with no feeding.

During the light/dark experiments, animals were contained in glass finger-bowls (350 ml) immersed in a water table with flowing sea water. Water in the bowls was changed twice daily. Fragments (1–2 mm^3) of fresh fish muscle were offered as food. In the light experiments, animals were exposed to a pair of Grolux fluorescent lamps (300 lumens/m^2) while dark bowls were covered with aluminum foil. The experimental light intensity chosen was within the range found by Gladfelter (1975) to elicit maximum lobate tentacle expansion. Randomly selected dark bowls were inspected under reduced light twice during the night and three times during the day, by briefly (<10 s) lifting the foil covering. Anemones were not observed to react to such brief exposure; Pearse (1974) reported a similar lack of response in the anemone *Anthopleura elegantissima* under the same treatment. Light bowls were inspected at two hourly intervals during the day and twice at night. As with the anemone *Anthopleura elegantissima* (Pearse, 1974), recording of expansion and contraction was simplified by the fact that the majority of anemones were either fully expanded or fully contracted under experimental conditions and arbitrary decisions were necessary only occasionally.

Results

Larvae and juveniles

Planulae larvae of *Lebrunia coralligens* were released at irregular intervals in May and June. A typical larva was 1 mm in length and 0.5 mm diameter at the apical

end. In the laboratory, larvae were negatively phototropic, showed aggregated settlement behavior, and settled most frequently around the bases of the adults. Up to 50 planulae were released from a single adult. The planulae contained zooxanthellae arranged in longitudinal rows, bore eight apical white patches (the precursors of the first set of tentacles), and could swim slowly in a spiral fashion.

Settlement and metamorphosis occurred in most cases within 24 h and Edwardsia stage juveniles with eight tentacles were produced. Additional juvenile tentacles subsequently developed in several rows and were intermittently expanded during both day and night. Lobate pseudotentacles did not develop until about six weeks later. A six-week development sequence is shown in Figure 1.

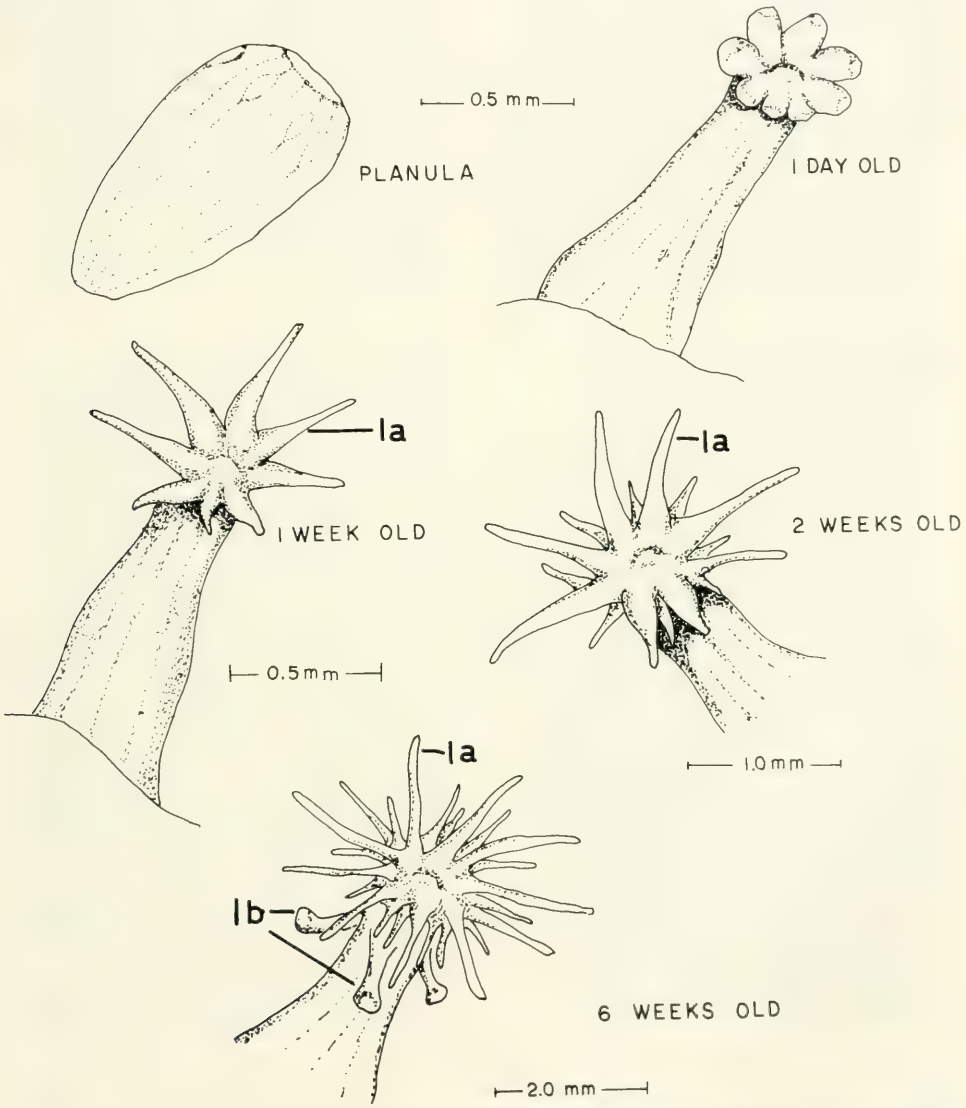

FIGURE 1. A six week development sequence of planula larva and juveniles of *Lebrunia coralligens*. 1a—lanceolate tentacle, 1b—lobate tentacle.

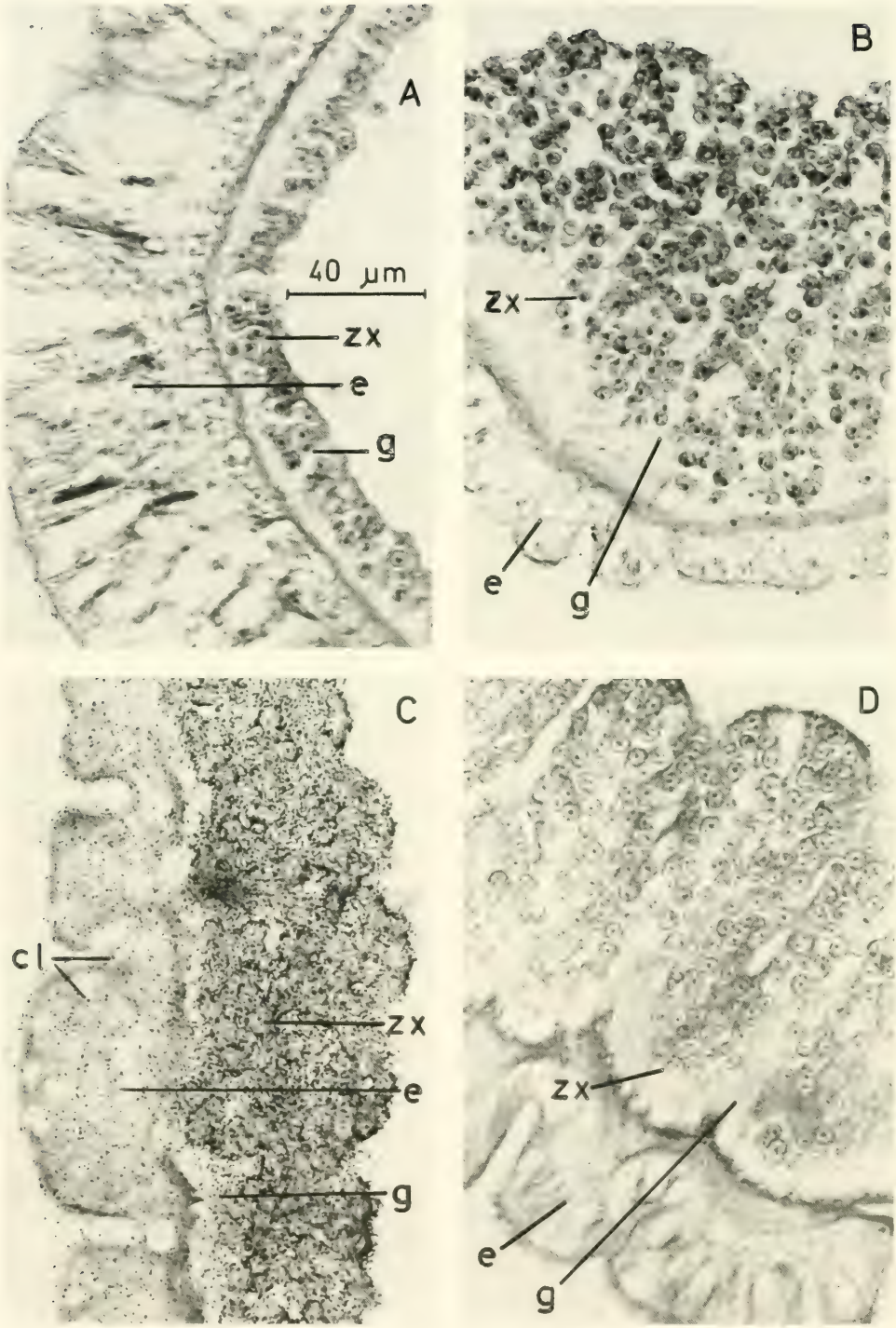

FIGURE 2. Cross sections of lanceolate feeding tentacles and lobate pseudotentacles of *Lebrunia coralligens*. A—section of lanceolate tentacle showing thin layer of zooxanthellae. B—section of lobate tentacle showing dense layer of zooxanthellae. C—autoradiograph of lobate tentacle incubated in light showing dense labelling by ^{14}C. D—autoradiograph of lobate tentacle incubated in the dark showing insignificant ^{14}C labelling. Same scale as shown in 2A throughout. ZX—zooxanthellae, e—epidermis, g—gastrodermis, cl—^{14}C label.

Respiration and photosynthesis

The relative abundance of zooxanthellae in the lanceolate and lobate tentacles of *Lebrunia coralligens* is shown in Figures 2A and B. In the lanceolate tentacles the cells are distributed in a thin layer while in the lobate tentacles they lie densely packed throughout a thick gastrodermal layer. When *L. coralligens* were incubated in $NaH^{14}CO_3$ in the light and analysed for incorporation of ^{14}C by tissue autoradiography it readily became apparent that uptake occurred in the zooxanthellae and in the tissue of the tentacles. Radioactivity associated with the zooxanthellae and the anemone tissue was high (Fig. 2C) after 12 h exposure. Control incubations in the dark showed that non-photosynthetic uptake of the ^{14}C by zooxanthellae was negligible (Fig. 2D).

Measurements of oxygen production in whole animals with expanded lobate tentacles showed a net oxygen production. In order to demonstrate that most of the photosynthetic activity occurs in the lobate tentacles, measurements of rates of oxygen production and of respiration were made on anemones in which all the lobate tentacles were first fully expanded and in which half of the tentacles were retracted. Contraction of lobate pseudotentacles was achieved by simply making a small incision in the tip of a tentacle with a pair of fine scissors. The incision caused the lobate tentacle to withdraw and contract. All lobate tentacles treated in this manner remained contracted for several days while uncut tentacles on the same animal were expanded normally. Thus, following the measurement of oxygen production and consumption in fully expanded animals, half of the lobate pseudotentacles on each specimen were incised and rates of production and consumption remeasured the next day. Results of measurements of rates of production and respiration of fully expanded and incised animals are shown in Table I. Production (P gross) rates were significantly higher in whole animals with fully expanded lobate tentacles than in incised anemones with reduced lobate tentacle expansion ($t = 3.21$, $P < 0.01$, from sets of paired observations). Respiration rates were not significantly different between the two groups, indicating that the incision treatment affected production rates only.

Diurnal cycles of tentacle expansion and contraction

The diurnal cycles of expansion and contraction of lobate and lanceolate tentacles of *Lebrunia coralligens* over a seven-day period are shown in Figures 3–5. Under a natural light program (Fig. 3A) less than 2% of the animals expanded their lobate tentacles in the dark and 92–100% were expanded in the light. During the first three

TABLE I

Mean rates (±S.D.) of oxygen production (P) and respiration (R) of whole and incised Lebrunia coralligens *(mg O_2/h)*

Whole animals[1] · (n = 20)		*Incised animals*[2] · (n = 20)	
P[3]	R[4]	P	R
2.94 (±0.63)	1.76 (±0.25)	1.94 (±0.23)	1.88 (±0.04)

[1] Animals with all lobate tentacles expanded.

[2] One half of the lobate tentacles in each animal were incised to induce lobate tentacle contraction.

[3] P is gross production, *i.e.*, 'apparent' production measured as increase in oxygen concentration in light vials plus measured respiration in the dark. [See McCloskey *et al.*, (1978) and Muscatine *et al.*, (1981) for definitions.]

[4] R was determined as decrease in oxygen concentration in darkened vials. Decrease in concentrations was 10–15% of initial values.

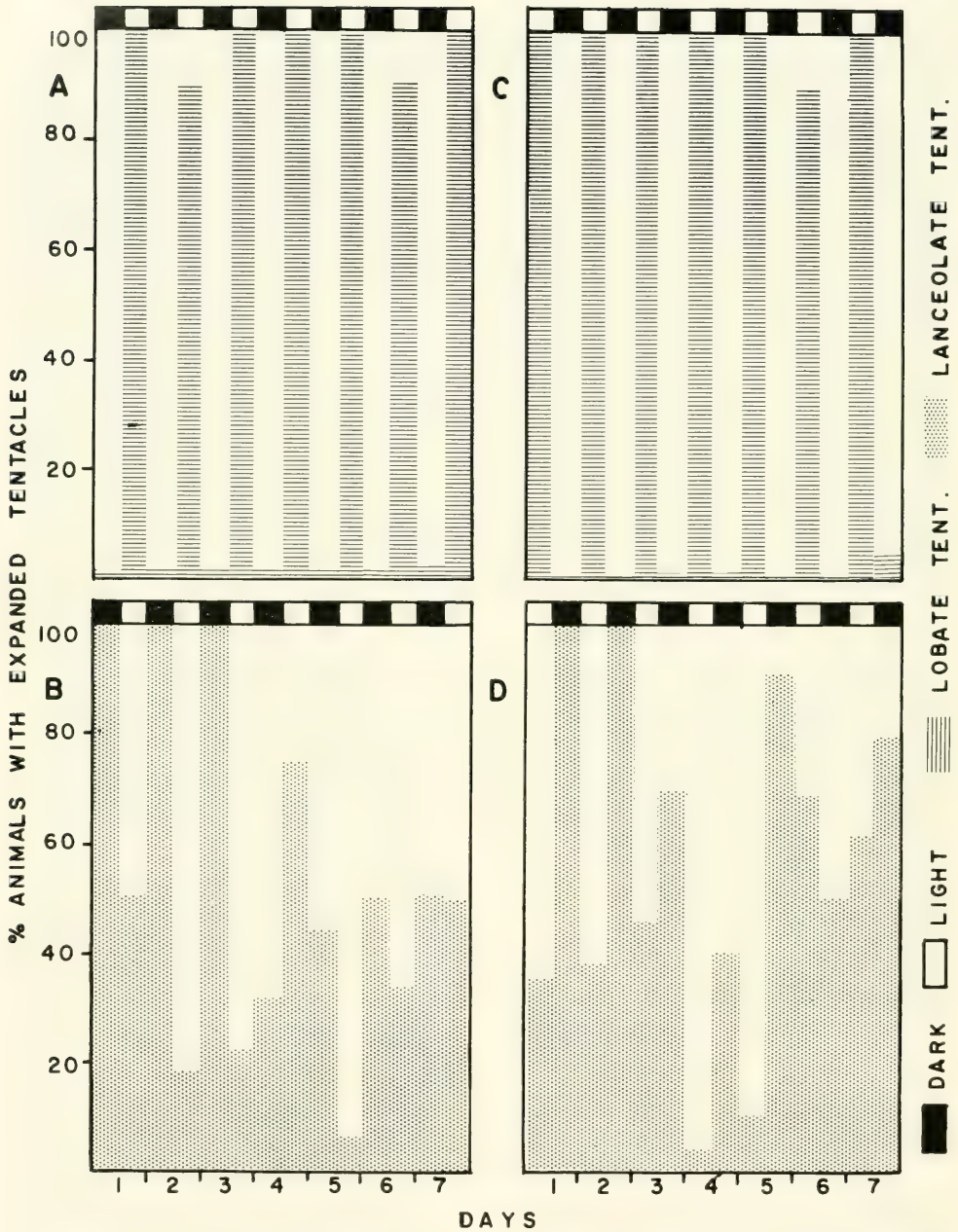

FIGURE 3. Expansion and contraction of tentacles of *Lebrunia coralligens* showing similar responses under natural and reverse 12 h cycles of light and darkness. Animals were fed on first and fourth days. Twenty animals in each group. A—diurnal rhythm of light expansion and dark contraction of lobate tentacles under natural 12 h cycle. B—diurnal rhythm of variable expansion and contraction of lanceolate tentacles under natural 12 h cycle. C—diurnal rhythm of light expansion and dark contraction of lobate tentacles under reverse 12 h cycle. D—diurnal rhythm of variable expansion and contraction of lanceolate tentacles under reverse 12 h cycle.

days lanceolate tentacles (Fig. 3B) were expanded in the dark in all animals but from the fourth through the seventh days lanceolate tentacles were expanded only in 30–50% of animals. In light conditions, the results were highly variable, with lanceolate tentacle expansion occurring in 6–75% of animals.

Under the reversed light cycle experiment (Fig. 3C), lobate pseudotentacles were fully expanded during the light periods in 100% of anemones with the exception of one period (90%). During the dark periods lobate pseudotentacles were fully contracted in 99% of individuals with the exception of one period (95%). Cycles of expansion and contraction of lanceolate tentacles under the reverse light cycle (Fig. 3D) were variable as for the normal cycle. During the first two days lanceolate tentacles were expanded in 100% of anemones in the dark and between 40 and 92% during the following days. Under light conditions expansion of lanceolate tentacles varied between 5 and 70% of anemones.

It is evident then that lobate pseudotentacles responded primarily to the absence or presence of light regardless of the time of day. Lanceolate tentacles were expanded in the dark during the first two or three days but cycles of expansion and contraction were variable thereafter.

In a second pair of experiments, two groups of unfed anemones were exposed to continuous light and continuous darkness for a one-week period (Fig. 4). In animals under continuous light (Fig. 4A) lobate pseudotentacles were expanded in 90 to 100% of animals throughout the seven-day period. Lanceolate tentacles were expanded in 43 to 93% of animals under the conditions of light exposure (Fig. 4B). Thus, as in the 12 h light/12 h dark experiments, the lobate tentacles responded consistently to the presence of light while the lanceolate tentacle response was positive but variable.

Under a regime of continuous darkness (Fig. 4C, D) over the seven-day period only 50% of the unfed animals survived beyond 2.5 days. Nevertheless during each two-day period of activity, lobate pseudotentacles were contracted in 95–98% of anemones and lanceolate tentacles expanded in 15–100% of specimens.

In order to determine the effect of feeding on tentacle behavior, two groups were fed daily with fragments of fish muscle (Fig. 5). Under conditions of continuous light (Fig. 5A) lobate pseudotentacles were fully expanded throughout the seven-day period in 70–100% of anemones, while lanceolate tentacles (Fig. 5B) were expanded in 60–100% of animals. Under conditions of continuous darkness (Fig. 5C, D) 50% mortality of fed anemones occurred every 2–2.5 days. Within each two-day period, lobate pseudotentacles remained 95–98% contracted while lanceolate tentacles were expanded in 10–100% of the animals.

In a comparison of fed and unfed groups of anemones under conditions of continuous light there were statistically significant differences between numbers of animals with expanded lobate and lanceolate tentacles. The number of fed animals with expanded lanceolate tentacles was greater than the number of unfed animals ($t = 3.27$, $P < 0.01$), while the number of fed animals with expanded lobate tentacles was less than unfed animals ($t = 3.23$, $P < 0.01$).

In continuous darkness the high mortality in both fed and unfed groups reduced the data base and prevented any statistical comparison of the two treatments. Lobate tentacles remained unexpanded in both fed and unfed groups while lanceolate tentacle expansion was highly variable.

Discussion

The lobate pseudotentacles of *Lebrunia coralligens* are photosynthetic organs and are similar to the enlarged bubble tentacles of the scleractinian coral *Plerogyra sinuosa*

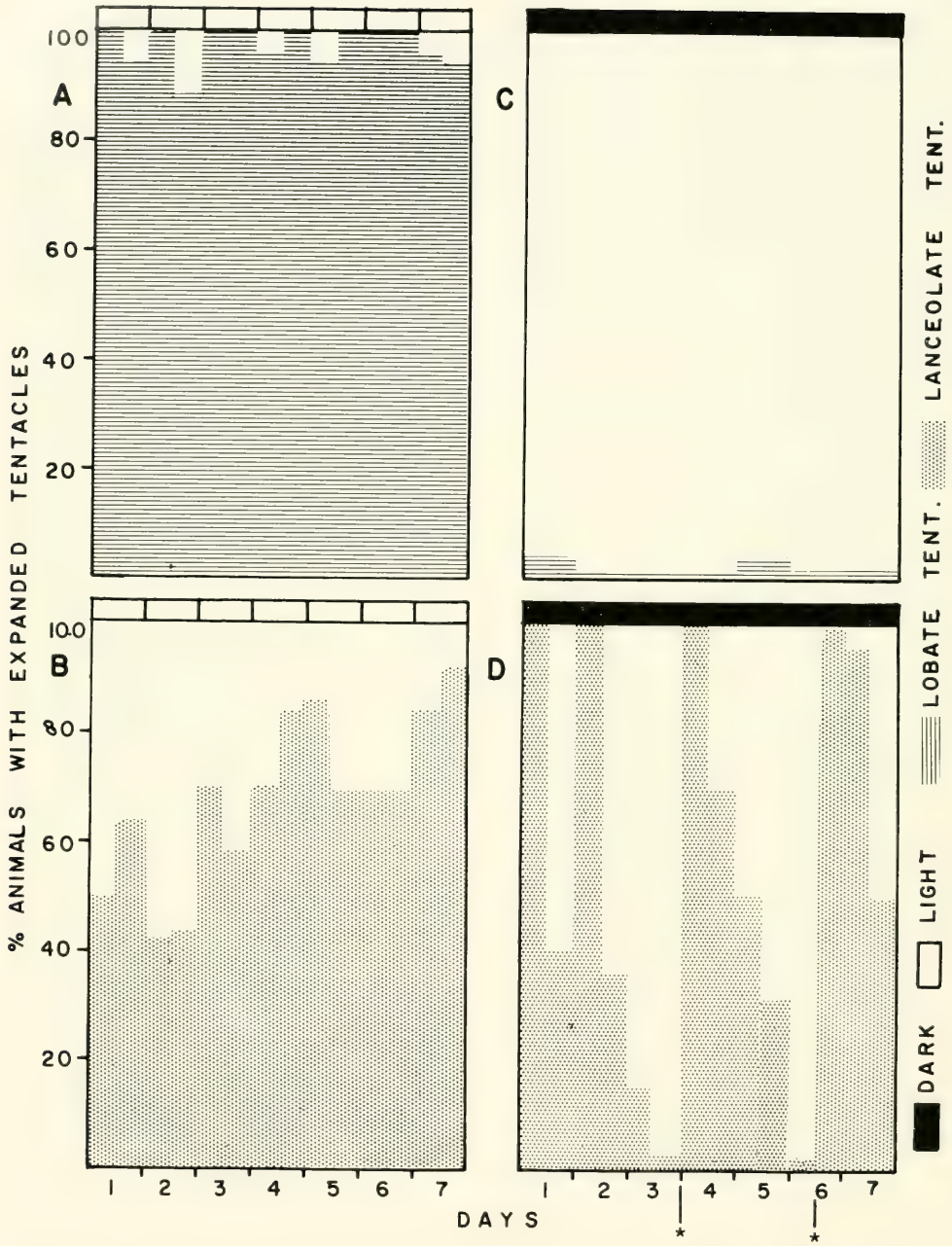

FIGURE 4. Expansion and contraction of the tentacles of unfed *Lebrunia coralligens* showing differences in responses under conditions of continuous light and continuous darkness. Twenty animals in each group. A—expansion of lobate tentacles under conditions of continuous light. B—expansion of lanceolate tentacles under conditions of continuous light. C—continuous contraction of lobate tentacles under conditions of continuous darkness. D—variable expansion and contraction of lanceolate tentacles under conditions of continuous darkness. Asterisks mark time when 50% of animals were dead and whole group replaced.

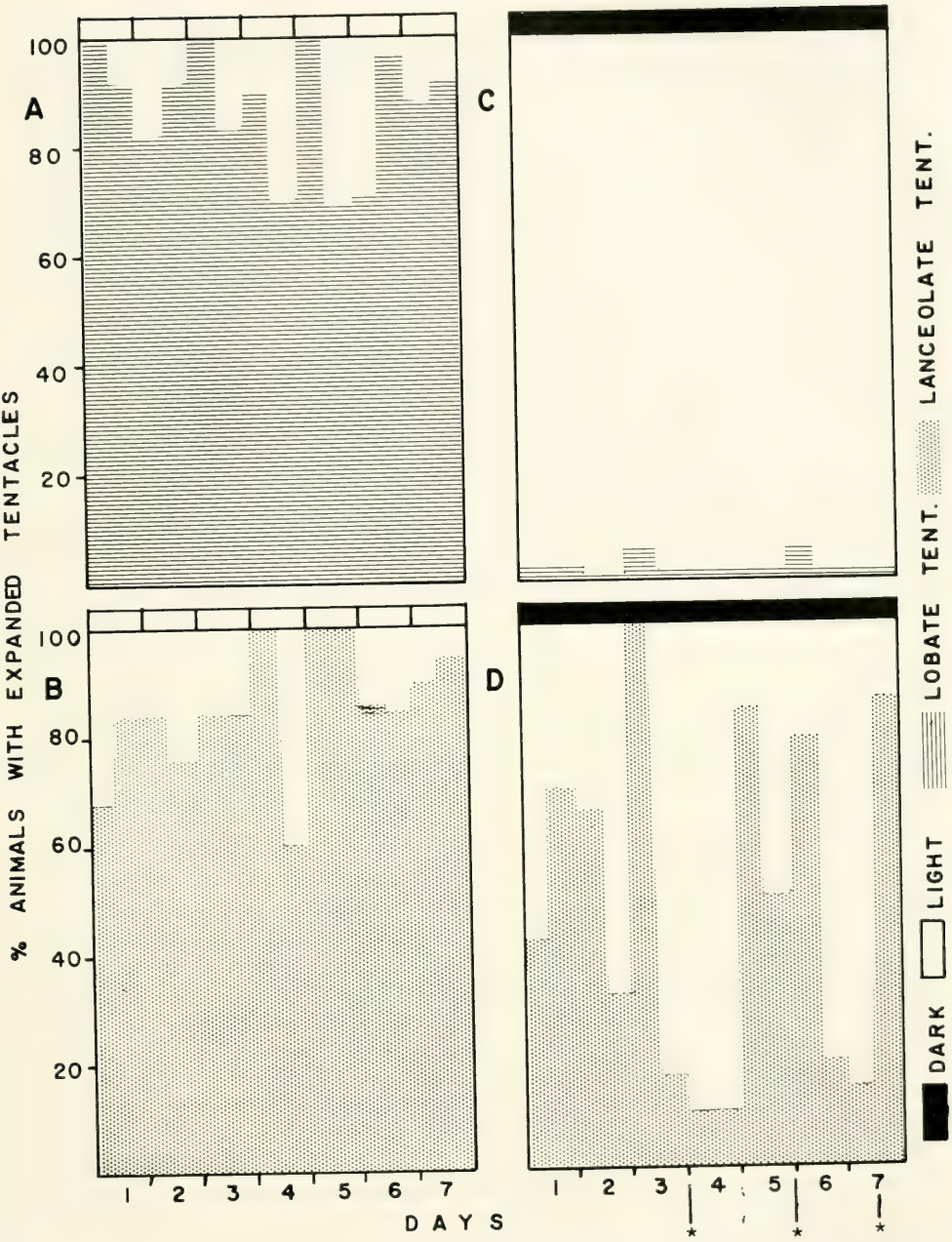

FIGURE 5. Expansion and contraction of the tentacles of *Lebrunia coralligens* fed daily, showing differences in responses under conditions of continuous light and continuous darkness. Twenty animals in each group. A—expansion of lobate tentacles under conditions of continuous light. Number of expanded animals significantly less than in Figure 4A ($t = 3.23$, $P < 0.01$). B—expansion of lanceolate tentacles under conditions of continuous light. Number of expanded animals significantly greater than in Figure 4B ($t = 3.27$, $P < 0.01$). C—continuous contraction of lobate tentacles under conditions of continuous darkness. D—variable expansion and contraction of lanceolate tentacles under conditions of continuous darkness. Asterisks mark the time when 50% of animals were dead and whole group replaced.

as reported by Fricke and Vareschi (1982). A mean gross production to respiration ratio (P/R) for a two hour midday period of 1.67 was determined for *L. coralligens*. This ratio, together with the evidence of translocation of carbon from zooxanthellae to animal tissue, suggests that the anemones may be capable of autotrophy (Muscatine *et al.*, 1981).

The dual nutritional strategy of *Lebrunia coralligens* appears to be reflected in a complex diel rhythm of expansion and contraction. Although sea anemones react to visible light in a variety of ways, the most frequently reported response is contraction (Pearse, 1974). However, a number of anemones which contain zooxanthellae are known to expand during the day and to contract at night (Gosse, 1860; Bohn, 1906; Gee, 1913; Smith, 1939; Pearse, 1974), while others with symbionts show a variety of responses (Pearse, 1974). Sebens and De Riemer (1977) have reported that two other anemones with auxilliary tentacles, *Lebrunia danae* and *Bunodeopsis antilliensis*, exhibit the same behavior as *L. coralligens*.

Both in the field (Sebens and De Riemer, 1977) and in the laboratory (Gladfelter, 1975), the lobate pseudotentacles of *Lebrunia coralligens* are expanded in the light and contracted in the dark. The same patterns of expansion and contraction under natural and reversed light/dark cycles in this study support the hypothesis of an exogenous rhythm of activity controlled by ambient light conditions. Furthermore, under the same laboratory conditions, but exposed to continuous light, the lobate tentacles remained expanded for a prolonged period of seven days.

However, light is apparently not the only factor involved, for under conditions of continuous light, anemones which were fed daily showed a significantly lower degree of expansion (number of animals with expanded lobate tentacles) than did anemones without food. This suggests that expansion of lobate tentacles is increased with a lowered nutritional state and decreased when the nutritional state rises above a certain threshold. Thus the unfed animals utilize their lobate tentacles so as to obtain the maximum nutritional intake by autotrophy while fed animals reduce their autotrophic intake.

While it is apparent that the lobate pseudotentacles of *Lebrunia coralligens* expand in the light and contract in the dark in a regular and precise way, the reverse rhythm does not always occur in the lanceolate tentacles. Although Sebens and De Riemer (1977) observed lanceolate feeding tentacles to be expanded at night and contracted during the day in the field, and Gladfelter (1975) found that light contraction and dark expansion would persist for 24 hours, the observed behavior in this study of lanceolate tentacles in the laboratory over a prolonged period was much more irregular and less predictable.

Under natural and reversed light/dark cycles, lanceolate tentacles were expanded in the dark during the first two or three days only and the numbers of expanded animals decreased thereafter. During the same experiment a considerable number of animals were also expanded in the light. Under conditions of continuous light many animals expanded their lanceolate tentacles over a seven-day period.

The variability of the contraction and expansion of the lanceolate tentacles suggests that their behavior is not entirely under the control of ambient light conditions, nor does it occur as a counter response to lobate tentacle condition. Lanceolate tentacles expand under conditions of both light and dark irrespective of whether the lobate tentacles are expanded or contracted. In juvenile *Lebrunia coralligens* without lobate tentacles, the lanceolate tentacles expanded intermittently in both light and dark. Gladfelter (1975) noted that lanceolate tentacles expanded when anemones were disturbed by removing them from the substrate.

Lanceolate tentacle expansion appears to be partly determined by food availability or nutritional state. Under continuous light conditions more fed animals had expanded lanceolate tentacles than did unfed animals. This is in contrast to the behavior of lobate tentacles in which feeding suppressed the degree of expansion. The stimulation of a feeding response by the presence of food in the water is well known in anemones (Reimer, 1970, 1971) and in reef corals (Mariscal and Lenhoff, 1968; Lewis and Price, 1975). The expansion of lanceolate tentacles at night as observed by Sebens and De Riemer (1977) is similar to the nighttime expansion of reef coral polyps (Porter, 1974) which occurs in response to the nighttime increase in plankton (Emery, 1968; Johannes *et al.*, 1970). Nevertheless, Goreau (1956) observed that some colonies of nearly all species of Atlantic reef corals are expanded during the daytime.

The high mortality of anemones under conditions of continuous darkness was an unexpected result. Although Muscatine (1961) found that the anemone *Anthopleura elegantissima* which contains zooxanthellae lost weight rapidly in continuous darkness, specimens remained alive for as long as 12 weeks. It is evident that light is necessary for the welfare of *Lebrunia coralligens* and possible that the lobate tentacle specialization increases the animal's light dependence.

It is concluded that the behavioral rhythms of the two types of tentacles are integrated by the nutritional status of the animal. The lobate tentacles are photosynthetic organs whose expansion is under the control of ambient light but may be modified by nutritional state. Lanceolate tentacle expansion on the other hand is primarily a feeding response. Thus anemone behavior is an expression of combined nutritional strategies.

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LITERATURE CITED

BOHN, G. 1906. Mouvements en relation avec l'assimilation pigmentaire chez les animaux. *C.R. Soc. Biol. Paris* **61:** 527–528.

EMERY, A. R. 1968. Preliminary observations on coral reef plankton. *Limnol. Oceanogr.* **13:** 293–303.

FRICKE, H., AND E. VARESCHI. 1982. A scleractinian coral (*Plesogyra sinuosa*) with "photosynthetic organs." *Mar. Ecol. Prog. Ser.* **7:** 273–378.

GEE, W. 1913. Modifiability in the behaviour of the California shore anemone, *Cribrina xanthogrammatica* Brandt. *J. Anim. Behav.* **3:** 305–328.

GLADFELTER, W. D. 1975. Sea anemone with zooxanthellae: simultaneous contraction and expansion in response to changing light intensity. *Science* **189:** 570–571.

GOREAU, T. F. 1956. A study of the biology and histochemistry of corals. Ph.D. Thesis, Yale University, New Haven.

GOSSE, P. H. 1860. *A History of the British Sea Anemones and Corals.* Van Voorst, London. 362 pp.

HYMAN, L. 1940. *The Invertebrates: Protozoa Through Ctenophora.* McGraw-Hill, New York. 726 pp.

JOHANNES, R. E., S. L. COLES, AND N. T. KUENZEL. 1970. The role of zooplankton in the nutrition of some scleractinian corals. *Limnol. Oceanogr.* **15:** 579–586.

LEWIS, J. B. 1960. The coral reefs and coral communities of Barbados, West Indies. *Can. J. Zool.* **38:** 391–435.

LEWIS, J. B., AND W. S. PRICE. 1975. Feeding mechanisms and feeding strategies of Atlantic reef corals. *J. Zool. Lond.* **176:** 527–544.

MARISCAL, R. N., AND H. M. LENHOFF. 1968. The chemical control of feeding behaviour in *Cyphastrea ocellina* and in some other Hawaiian corals. *J. Exp. Biol.* **49:** 689–699.

McCloskey, L. R., D. S. Wethey, and J. W. Porter. 1978. Measurement and interpretation of photosynthesis and respiration in reef corals. Pp. 369–379 in *Coral Reefs: Research Methods,* D. R. Stoddart and R. E. Johannes, eds. Monographs on Oceanographic Methodology, 5, UNESCO, Paris.

Muscatine, L. 1961. Symbiosis in marine and freshwater coelenterates. Pp. 225–268 in *The Biology of Hydra and of Some Other Coelenterates,* H. W. Lenhoff and W. F. Loomis, eds. Univ. of Miami Press, Coral Gables, FL.

Muscatine, L., L. R. McCloskey, and R. E. Marian. 1981. Estimating the daily contribution of carbon from zooxanthellae to coral animal respiration. *Limnol. Oceanogr.* **26:** 601–611.

Pearse, V. B. 1974. Modification of sea anemone behaviour by symbiotic zooxanthellae: expansion and contraction. *Biol. Bull.* **147:** 641–651.

Porter, J. W. 1974. Zooplankton feeding by the Caribbean reef-building coral, *Montastrea cavernosa. Proc. Second Int. Symp. Coral Reefs, Great Barrier Reef Committee, Brisbane* **1:** 111–125.

Reimer, A. A. 1970. Chemical control of feeding behaviour and role of glycine in the nutrition of *Zoanthus* (Coelenterata, Zoanthidea). *Comp. Biochem. Physiol.* **39A:** 743–759.

Reimer, A. A. 1971. Feeding behaviour in the Hawaiian zoanthids *Palythoa* and *Zoanthus. Pac. Sci.* **25:** 512–520.

Sebens, K. P., and K. De Riemer. 1977. Diel cycles of expansion and contraction in coral reef anthozoans. *Mar. Biol.* **43:** 247–256.

Smith, H. G. 1939. Relationship between actinians and zooxanthellae. *J. Exp. Biol.* **16:** 334–345.

Strickland, J. D. H., and T. R. Parsons. 1965. A manual of sea water analysis. *Bull. Fish. Res. Bd. Can.* **167:** 311 pp.

Wilson, H. V. 1890. On a new Actinia, *Haplophoria coralligens. Johns Hopkins Univ. Stud. Biol.* **4:** 379–387.

Reference: *Biol. Bull.* **167**: 613–629. (December, 1984)

PATTERNS OF WHOLE COLONY PREY CAPTURE IN THE OCTOCORAL, *ALCYONIUM SIDERIUM**

MARK R. PATTERSON

Biological Laboratories, Harvard University, 16 Divinity Avenue, Cambridge, Massachusetts 02138

ABSTRACT

Colonies of a boreal octocoral, *Alcyonium siderium,* preferentially catch prey on specific regions of the colony at certain flow speeds of low turbulence. Colonies feeding on brine shrimp cysts capture prey preferentially on the upstream side of the colony under low flow conditions (2.5 $cm \cdot s^{-1}$). At intermediate flow speeds (9.0 $cm \cdot s^{-1}$), prey capture is uniformly distributed around the circumference of the colonies, while at higher flow speeds (19.0 $cm \cdot s^{-1}$), prey capture again becomes asymmetric and downstream polyps capture the most prey. At higher levels of free-stream turbulence, these asymmetric prey capture distributions around the colony disappear; in the vertical direction, prey capture is asymmetric over the surface of the colony at all flow speeds tested, with polyps nearer the top of the colony capturing the most prey only at the lowest speeds. Asymmetrical filtration results from (1) increasing mechanical deformation of polyps into an orientation unfavorable for prey capture with increasing flow speed, and (2) differential prey concentrations in the boundary layer of the colony in the downstream direction. For non-motile particles, the filtration performance of this passive suspension feeder appears governed only by the flow speed and turbulence, the mechanical behavior of the filter elements, and the motion of the particles in the boundary layer of the colony.

INTRODUCTION

Organisms which rely on ambient water currents to bring them suspended particulate food are termed passive suspension feeders (Jørgensen, 1955) and are often the most conspicuous members of benthic communities. Passive suspension feeding is especially common in the phylum Cnidaria. Coral reef cnidarians may require periodic inputs of zooplankton to meet their nutritional requirements (Johannes *et al.,* 1970; Muscatine and Porter, 1977). Boreal cnidarians such as sea anemones, octocorals, and ahermatypic scleractinians rely almost exclusively on passive suspension feeding in obtaining food. Success in catching particulate prey can determine body size and mode of reproduction in sea anemones (Purcell, 1977; Sebens, 1981; Sebens and Koehl, 1984), and colony size in an octocoral (Sebens, 1984). Since passive suspension feeders are sessile, they must contend with potentially damaging hydrodynamic drag forces while filtering the water for food (Wainwright and Koehl, 1976).

Orientation to ambient currents can occur in passive suspension feeders as a result of their sessile habit. Cnidarians such as hydroids (Riedl, 1971; Svoboda, 1976) and octocorals (Théodor and Denizot, 1965; Grigg, 1972; Rees, 1972; Patterson, 1980) often orient the longest dimension of the colony perpendicular to the prevailing

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Abbreviations: K-S, Kolmogorov-Smirnov.

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flow. Such an arrangement permits the colony to intercept the greatest amount of prey per unit time (Warner, 1976). Maximization of food intercepted by prey-capturing surfaces may underly the behavioral ecology of crinoids (Meyer, 1973; Macurda and Meyer, 1974), and ophiuroids (Warner, 1971; Warner and Woodley, 1975), as well as sea anemones (Robbins and Shick, 1980). Hermatypic scleractinians also display orientation to currents and surge. In some species, the shape of the colony enhances water flow through the branches (Abbott, 1974); in others, orientation minimizes the bending stresses in the larger branches of the colony (Chamberlain and Graus, 1975; Vosburgh, 1977).

Orientation to flow is a well-studied phenomenon in passive suspension feeders, yet few studies have examined how prey capture occurs at either the polyp or whole colony level. While the prey caught in the field by passive suspension feeders have been described and quantified [*e.g.*, corals (Porter, 1974), zoanthids (Sebens, 1977), sea anemones (Purcell, 1977; Sebens, 1981), soft corals (Sebens and Koehl, 1984)], there are few quantitative descriptions of prey capture mechanisms (Koehl, 1977; Lasker, 1981). A step towards a quantitative theoretical understanding of how biological filters work was taken by Rubenstein and Koehl (1977). They suggested that the engineering theory of aerosol filtration could serve as a possible unifying scheme for biological filtration. They demonstrated that all biological filters need not act like simple sieves as previously tacitly assumed, but that there are at least five possible mechanisms for particle capture at the level of the filtering element. They also showed how, for simple filter element geometries, the theory allowed predictions of filtration efficiencies for the different mechanisms. LaBarbera (1978) subsequently applied the theory of aerosol filtration to passive suspension feeding in an ophiuroid and found excellent agreement between his results and the predictions of particle collection efficiency calculated using the theory. However, a more recent review (Jørgensen, 1983) of the applications of aerosol filtration theory to suspension feeding indicates that more work needs to be done on determining the limits of applicability of some of the concepts, especially on how direct interception functions as the dominant mode of particle capture (LaBarbera, 1984). The role of particle loss after capture and the surface properties of biological filters are liable to be the areas which can explain the deviations of theory from observation.

How important are the spatial arrangements of *assemblages* of filter elements (*i.e.*, colonies or tentacle crowns), in determining local flow patterns? What effect does flow over a filter have on the motion of a passive food particle in the boundary layer of the organism? Patterns of flow around benthic invertebrates have only recently been studied in scleractinians (Chamberlain and Graus, 1975), hydroids (Svoboda, 1976), sand dollars (O'Neill, 1978; Telford, 1981), brachiopods (LaBarbera, 1981), and zoanthids (Koehl, 1977), and have been shown to have important implications for feeding behavior and shape in these organisms. Leversee (1976) studied whole-colony feeding in gorgonians and assumed the same first order feeding rate model previously used to describe *active* suspension feeding (Jørgensen, 1949). His study clearly demonstrated the importance of *in situ* orientiation to prevailing currents, and indicated the asymmetric nature of food capture (downstream side of colony). Capture of prey on the downstream side of the colony has also been observed by Lasker (1981) in gorgonians and by Porter (1979) in scleractinians. Okamura (University of California, Berkeley, pers. comm.) recently discovered that success in prey capture by individual zooids in an erect bryozoan is dependent on both location on the colony and flow speed. But in general, patterns of prey capture of sessile suspension feeders have not been investigated under controlled experimental conditions, and thus it is difficult to interpret the few patterns that have been reported.

A passive suspension feeder which is locally abundant in the New England subtidal is the colonial soft coral, *Alcyonium siderium* Verrill, a zooplanktivore (Sebens and Koehl, 1984). Little is known about it other than work on gametogenesis (Feldman, 1976), larval ecology (Sebens, 1982, 1983a, b, c), and the composition of prey taken in the field (Sebens and Koehl, 1984). Colonies often have simple geometries [spheroids, ellipsoids, cylinders; Patterson (1980)] which simplifies many experiments addressing flow patterns and locations of prey capture. Colonies also occur on shells of the horse mussel, *Modiolus modiolus,* and this facilitates their use in laboratory feeding experiments. Prey capture of zooplankton in the field and laboratory can be observed with the naked eye as the tentacles of this species measure approximately 3 mm (Sebens and Koehl, 1984) and contract in a characteristic fashion toward the pharynx after catching a zooplankton. It was possible to measure success in prey capture over colony surfaces after short feeding bouts in the laboratory, and to quantify the effect of flow speed and turbulence intensity on this process.

Materials and Methods

All laboratory experiments were conducted at the Marine Science and Maritime Studies Center (MSMSC), Northeastern University, Nahant, Massachusetts, where colonies of *Alcyonium siderium* were collected monthly and maintained in flowing sea water tables. A recirculating flow tank was constructed from plexiglas, patterned after a design published by Vogel and LaBarbera (1978) and was used in feeding experiments. Center channel midwater flow speeds in the working section [12 cm (depth) × 16 cm (width) × 75 cm (length)] were continuously variable between 0 and 40 $cm \cdot s^{-1}$. Volume of the system when full was about 98 l. Shear velocity (U*) was calculated by regressing average flow speed at a height (z) *versus* ln z, and could be set as high at 1.6 $cm \cdot s^{-1}$ if no turbulence reducers (Hexcel flow straighteners, cell diameter = 1.0 cm) were used. When the Hexcel flow straighteners were removed from the upstream section of the tank work area, the level of turbulence increased and mean flow speed profiles became steeper, approximating the hydraulically rougher flow seen by these organisms in the field.

The impeller used to push water through the tank was driven by a Bodine NSH-12 1/50 hp DC motor controlled by a Bodine ASH-500 solid state speed control. Variation in motor speed was less than 2%. Sea water was obtained from the MSMSC sea water system and was filtered twice (sand, cotton mesh) to remove particles greater than 20 μm in diameter.

Flow speeds and turbulence intensities (see Bradshaw, 1971, for a discussion of turbulence and its measurement) were measured with a two channel thermistor flowmeter circuit (LaBarbera and Vogel, 1976). This device was contained in an underwater housing (Ikelite #4610) for *in situ* field measurements. Data were recorded as an FM signal on a microcassette tape recorder (Olympus Pearlcorder S802) with high-quality microcassette (Olympus MC-90-AR); a tape speed of 1.2 $cm \cdot s^{-1}$ allowed continuous recordings of up to two hours in the laboratory and field. Recordings were read from the tape into an Apple IIe microcomputer via a signal conditioner (custom-made) and 8 bit successive approximation A/D convertor (Mountain Computer) at a sampling rate of 10 Hz. Seven points were used in the regression calculations of shear velocity using ensemble averages. Flow speed profiles and turbulence intensity were measured 10 cm upstream of the colonies tested (see Fig. 2).

Mussels (*Modiolus modiolus*) with octocoral colonies were collected from the subtidal using SCUBA. In the laboratory, the mussel shells were fractured, and shell fragments with single colonies were cut using tin snips.

Alcyonium readily catches and ingests hydrated *Artemia* cysts. Hydration of desiccated cysts occurs in less than 30 minutes (hydrated mean diameter 210 ± 15 μm S.D.; n = 100), and they were almost neutrally buoyant. Cysts did not change size significantly during the first four hours of use (*t*-test; $P < 0.05$; n = 100). The cysts are within the size range preferred by *Alcyonium* in the field (Sebens and Koehl, 1984). They were easily seen in the transluscent coelenteron of polyps for up to 12 hours after capture.

Location of prey capture

The spatial position of prey capture on colonies with bilaterally symmetric geometries was studied in flows of low and high turbulence levels and differing mean flow speeds. Colonies ranged in size from 2 to 8 cm in greatest dimension when expanded and generally approximated bilaterally symmetric ellipsoids and spheroids. Prior to the start of a feeding bout, a single colony was introduced to the flow tank and allowed to acclimate to the flow regime selected for the experiment and to expand its polyps fully. A standard volume of sea water (1.5 l) and hydrated cysts were added to the flow tank all at once at the start of the bouts which lasted 10 minutes. About five minutes into the experiment, three 60 ml samples were withdrawn isokinetically (Parker, 1968; Hewitt, 1978) from the flow using a Cole-Parmer peristaltic pump (model no. 7568) smoothed with hydraulic capacitors. Tests indicated that appreciable settling of the cysts did not occur throughout the duration of the experiment. Samples were filtered onto gridded Millipore filters, the number of cysts was counted, and a mean concentration of particles present in the flow was calculated.

At the end of each feeding bout, the flow was stopped and capture sites of cysts on the colony were mapped. Side projections and censuses were made by moving the colony to the side of the flow tank and mapping capture sites directly on the clear plexiglas tank wall with a felt-tip pen. The maps were then transferred to tracing paper; this side view mapping was only performed for feeding experiments with the flow straighteners removed. Top projections of colonies were made by removing colonies from the tank and transferring them to a small bowl. The top projection of the colony and location of caught particles were traced on a clear plastic sheet suspended over the bowl. The transfer of the colony out of the tank did not result in the loss of any prey items already inside the coelenteron. This procedure necessarily missed particles on the underside of the colonies in top projections and the backside of colonies in side projections. Figure 1 shows a typical top and side view of a colony after a feeding bout, with the coordinate system used to assess location of capture.

Areas of projected colony surfaces were measured using an Apple Graphics Tablet digitizer with a linear resolution of 0.1 mm. The digitizing tablet was also used to locate the centroid of top projections, and an angular coordinate system for these projections was established using the centroid and direction of flow past the colony (see Fig. 1). Twelve angular sectors of 30 degrees each were established for each colony and the data for the bilaterally symmetric halves were pooled for analysis. Side projections were divided into five non-dimensional length sectors (see Fig. 1). Particle counts in each angular or length sector were normalized by the projected total area available for particle capture.

Polypal deflections in flow

The deflections of individual polyps at various angular positions around a colony surface in different flow regimes were measured by one of two methods. Deflections

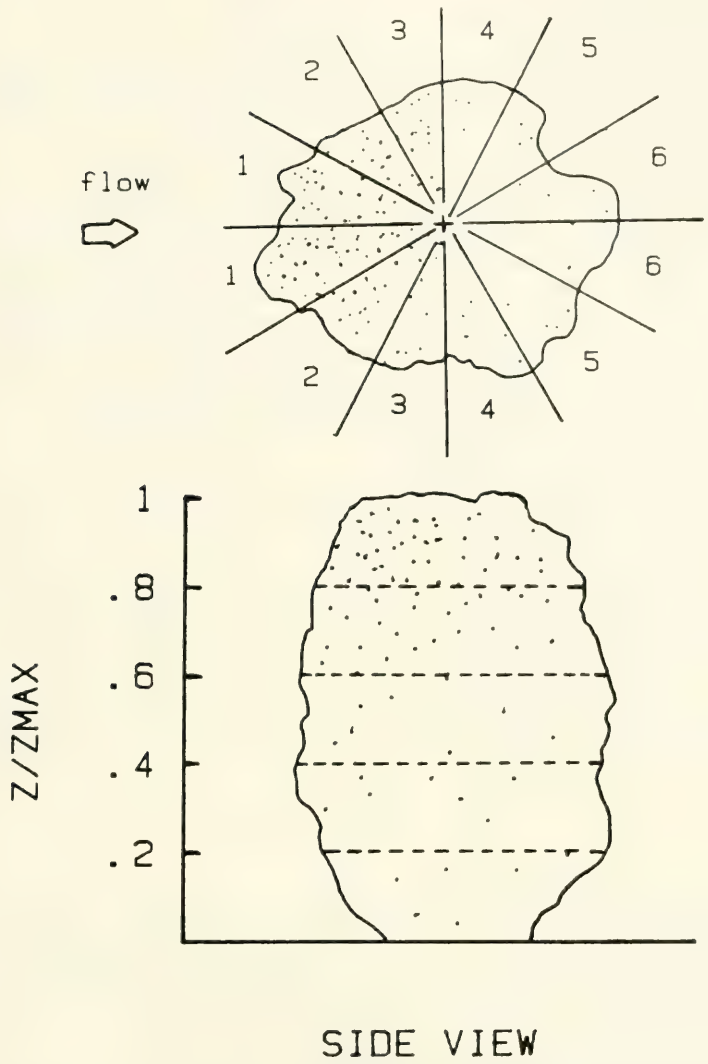


FIGURE 1. Particle capture sites on a typical *Alcyonium* colony. The upper figure is a vertical projection divided into bilaterally symmetric 30 degree sectors, with 0 degrees upstream and 180 degrees downstream. The dots indicate sites of prey capture, the cross indicates the centroid of the projected area in the top view, and the arrow indicates the direction of flow. The lower figure is a lateral projection showing the dimensionless five sector height coordinate used.

at lower speeds were measured directly through a dissecting microscope with micrometer ocular suspended above the flow tank. At the higher flow speeds tested, deflections were measured on tracings made from films of colonies in flow. Films were made using a Minolta XL 401 Super 8 mm movie camera with macro lens and were single-frame projected onto tracing paper. Deflections were measured to the nearest 0.1 mm with either scheme. In both types of experiments, polyps were carefully chosen to be those deflecting in a plane parallel to the focal plane of the camera.

Boundary layer particle concentrations

Concentrations of particles in the boundary layer of the colony are difficult to measure without employing sophisticated and expensive techniques such as laser-doppler anemometers (Lee and Srinivasan, 1978; Bunimovich and Kudin, 1982). However, I measured particle concentrations integrated over periods of minutes at various angular positions around a colony using a dissecting microscope suspended over the working section of the flow tank. A magnification of 50X was used to observe the passage of cysts through a specified volume surrounding individual polyps (Fig. 4A). This cylindrical volume had a radius of 2.0 tentacle lengths and a height equal to the anthocodial stem height plus the tentacle height. Focal distance and magnification were adjusted so that the depth of field when checked with a printed scale on a ruler was equal to the diameter of the cylindrical volume. During timed 180 second intervals (T), particles in focus passing through the volume were counted (N). Thermistor flowmeter measurements of speed (U; $cm \cdot s^{-1}$) were then made at these same locations at the level of the tentacular surfaces. An estimate of the expected number of particles (Ne) was calculated for each station, where $Ne = A \times U \times C \times T$, with C = ambient cyst concentration ($particles \cdot cm^{-3}$), and A = projected surface area of the volume (cm^2). All these measurements were made at a flow speed of less than 5 $cm \cdot s^{-1}$.

At higher flow speeds (>5 $cm \cdot s^{-1}$), it was not possible to determine reliably which particles were in focus and a different technique was used to estimate particle availability. Collimated beams of light were passed immediately upstream and downstream of the colony in flow in a darkened room and short time exposure photographs (1/8 s) taken. Cylindrical volumes were established and particle streaks beginning inside the volume were counted. Hence particle concentrations could be established directly using this technique. A check of this technique at low speeds indicated it gave the same results as those obtained using direct observations.

Results

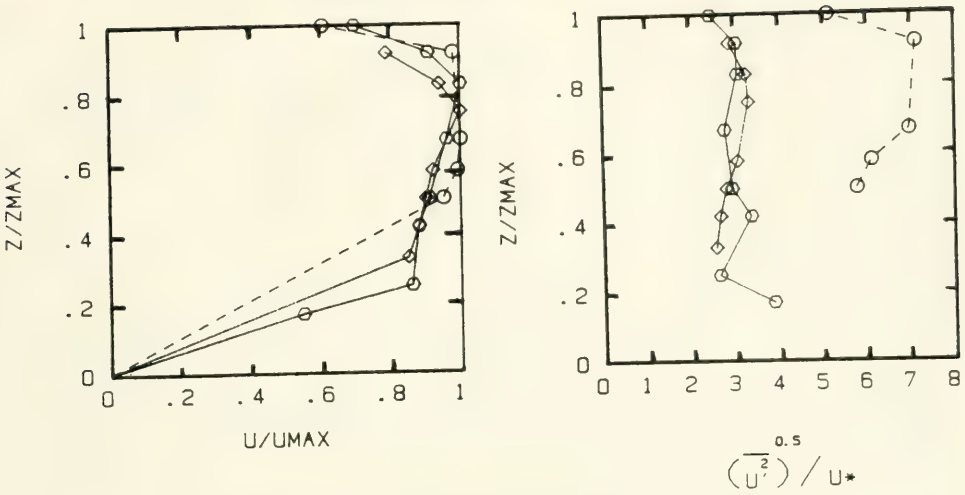
Feeding experiments: low turbulence

Experiments were first conducted in flows that did not show steep speed profiles or have high turbulent kinetic energy levels. The flow became hydraulically 'rougher' (Nowell and Church, 1979) when the flow straighteners were removed (see Fig. 2). Examination of patterns of particle capture for a colony in the less rough flow may offer insights into feeding observed in a hydraulically rougher flow.

Figure 3 depicts the results of experiments conducted at mean maximum flow speeds of 2.5, 9.0, and 19.0 $cm \cdot s^{-1}$. Percent data were arcsine transformed before calculation of statistics (Zar, 1974), and then back-transformed for graphical portrayal. Angular position of capture is markedly asymmetric at the lowest speed, with upstream polyps capturing significantly more prey than their downstream neighbors [Kolmogorov-Smirnov (K-S) goodness of fit, $P < 0.01$]. At intermediate flow speeds, the distribution of prey cannot be distinguished from an even distribution (K-S, $P > 0.10$), while at the highest flow speed tested, an asymmetric distribution again appears, with downstream polyps enjoying greater success in prey capture (K-S, $P < 0.01$).

Boundary layer experiments

Boundary layer measurements of particle availability in the downstream direction over a colony are shown in Figure 4B. At the flow speeds tested (<5 $cm \cdot s^{-1}$ at a



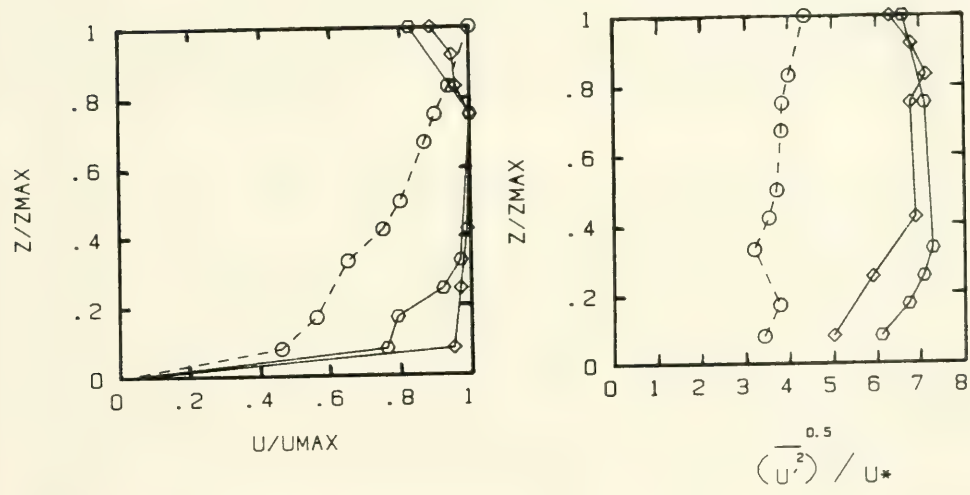


FIGURE 2. Flow regimes encountered by *Alcyonium* colonies in the recirculating flow tank with and without turbulence reduction. Left-hand graphs show flow speed (U) profiles in a direction normal to the substrate, normalized to the maximum mean flow speed (UMAX). Right-hand graphs depict turbulent kinetic energy ($\overline{U'^2}$) in the direction normal to the substrate, normalized to the shear velocity (U^*). Height above the substrate (Z) is normalized to the depth of the working section of the flow tank (ZMAX = 12.0 cm). Note the effect of the flow straighteners on the steepness of the flow profile and the level of turbulence in the flow tank. With flow straighteners: circles (UMAX = 2.5 $cm \cdot s^{-1}$; U^* = 0.2 $cm \cdot s^{-1}$), hexagons (UMAX = 9.0 $cm \cdot s^{-1}$; U^* = 1.0 $cm \cdot s^{-1}$), diamonds (UMAX = 19.0 $cm \cdot s^{-1}$; U^* = 1.6 $cm \cdot s^{-1}$). Without flow straighteners: circles (UMAX = 2.7 $cm \cdot s^{-1}$; U^* = 0.3 $cm \cdot s^{-1}$), hexagons (UMAX = 12.2 $cm \cdot s^{-1}$; U^* = 0.5 $cm \cdot s^{-1}$), diamonds (UMAX = 19.8 $cm \cdot s^{-1}$; U^* = 1.5 $cm \cdot s^{-1}$).

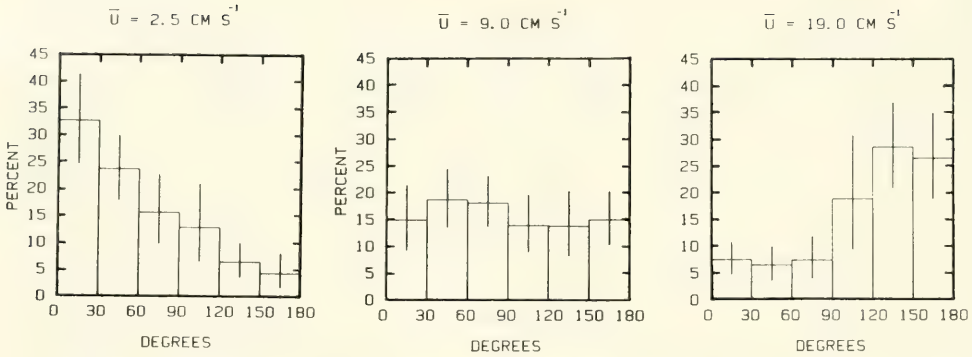


FIGURE 3. Angular position of prey capture events for low turbulence flow at three different flow speeds. Data from the bilateral halves of the colony are pooled; hence the abscissa runs from 0 to 180 degrees, with 180 degrees being the extreme downstream point. Capture frequencies have been normalized relative to the amount of area available for prey capture. Vertical bars are 95% confidence intervals. For flow speeds of 2.5, 9.0, and 19.0 cm·s^{-1}, the number of colonies fed were 12, 13, and 11, and the total number of cysts caught were 283, 425, and 186, respectively.

height of 5 cm), the concentration of cysts in the boundary layer of the four colonies tested showed a highly significant reduction in the downstream direction. Results of collimated light beam measurements are shown in Table I. At higher flow speeds (>9.0 cm·s^{-1} at a depth of 5 cm), this asymmetry in particle concentration disappeared. Concentrations of particles in the wake were no lower than those of the free-stream

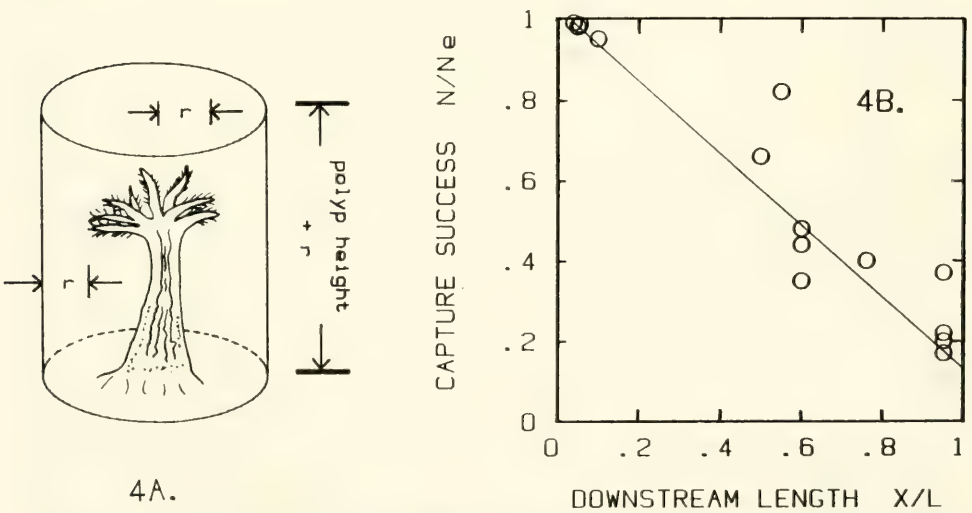


FIGURE 4. Boundary layer depletion measurements. (A) Volume used in boundary layer studies of feeding. Dimensions of the volume are indicated in the upper figure with r = radius of the oral disk. (B) Particles passing through the control volume were counted (N), and compared with an expected value, Ne = A × U × C × T, where A = projected cross-sectional area of control volume (cm^2), U = mean flow speed 2r above the tentacles (cm·s^{-1}), C = ambient particle concentration (no.·cm^{-3}), and T = counting period (s). The downstream length coordinate (X) used to locate the position of polyps was measured in a curvilinear fashion around the perimeter of the colony, and was normalized by the overall length coordinate (L) measured in the same fashion. The equation of the line fit by least-squares method to the data is N/Ne = −0.89 (X/L) + 1.03 with R^2 = 0.88. T = 180 seconds.

TABLE I

Mainstream versus *wake concentrations of* Artemia *cysts near a colony of* Alcyonium *as function of flow speed*

U ($cm \cdot s^{-1}$)	Delta concentration
0.9	−0.08 (0.22)
5.6	0.04 (0.22)
7.3	0.04 (0.47)
8.4	0.07 (0.41)
9.5	0.10 (0.27)

Concentrations measured photographically using beams of light immediately upstream and downstream of the colony. A positive mean delta C indicates that the concentration in the wake was higher than that in the mainstream. Values given are mean (S.D.) and have been normalized by the mean mainstream concentration during the experiments (414 $cysts \cdot l^{-1}$). All values are not significantly different from zero (t-test, $P < 0.01$) indicating that concentrations of particles in the wake and mainstream were the same.

(Table I); in fact, they were slightly higher, although this difference was not significant (t-test, $P < 0.01$).

Table II demonstrates that polypal deflections are greatest towards the upstream side of the colonies tested, with polyps in the wake experiencing little or no deflection. Deflections were greatest at the 90° angular position when the flow speed was strong enough to deflect enough upstream polyps out of the way of the flow. Figure 5 shows a profile of polyp movements from an experiment where the flow was increased from 0 to 8.4 $cm \cdot s^{-1}$ over a small colony.

Feeding experiments: higher turbulence

The pattern of particle capture observed in a hydraulically rougher flow is shown in Figure 6. Note that the distribution of capture events at the three mean maximum flow speeds tested (2.7, 12.2, 19.8 $cm \cdot s^{-1}$) has lost the asymmetry seen in the less turbulent case. Distributions could not be distinguished from an even distribution of prey around the colony (K-S, $P > 0.10$).

In the vertical direction, there was a significant tendency for colony sectors furthest from the substrate to catch relatively more prey at low speeds (Fig. 7) than sectors closer to the tank floor; at higher speeds, the distribution flattened out and then became significantly different from an even distribution at the highest flow speed tested (K-S, $P < 0.05$), showing a bimodal distribution with polyps at intermediate height on the colony catching the least amount of prey on a per capita basis.

TABLE II

Polypal deformation (d/L) versus *flow speed (U)*

	Angular position (degrees)		
U ($cm \cdot s^{-1}$)	0	90	180
2.5	0.01 (0.02)	0.09 (0.07)	0 (0)
9.0	0.43 (0.42)	0.95 (0.08)	0 (0)
19.0	0.50 (0.16)	0.67 (0.17)	0 (0)

Angular position stations are measured relative to the centroid of the colony when viewed from above. Polyps in the wake moved back and forth in the turbulence, but had no net displacement. Values given are mean (stan. dev.). n = 8 polyps per station. d = polyp tip deflection, L = polyp length.

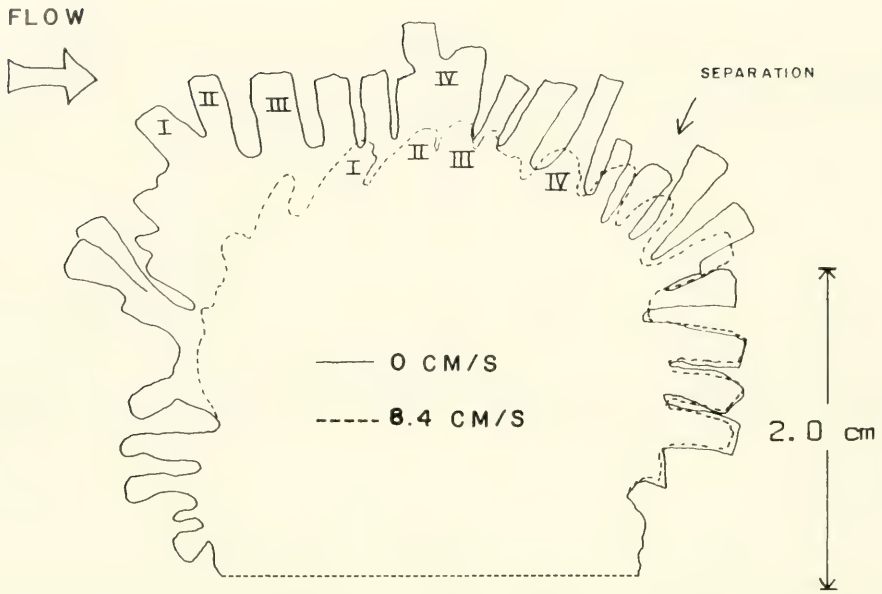


FIGURE 5. Deformation of polyps on the surface of a small colony is visible in this tracing made from a Super 8 mm film of the colony in still water and then in a flow of 8.4 $cm \cdot s^{-1}$ measured at the level of the height of the colony 10 cm upstream. Note the lack of deflection of the polyps in the wake of the colony. The separation point was determined using a filament of dye.

DISCUSSION

Flow in nature

Colonies of *A. siderium* typically occur on shallow (<20 m depth) subtidal vertical rocky substrates with some colonies occurring on the undersides of boulders, on horizontal rock surfaces, and on *Modiolus* shells attached to rock. The flow envi-

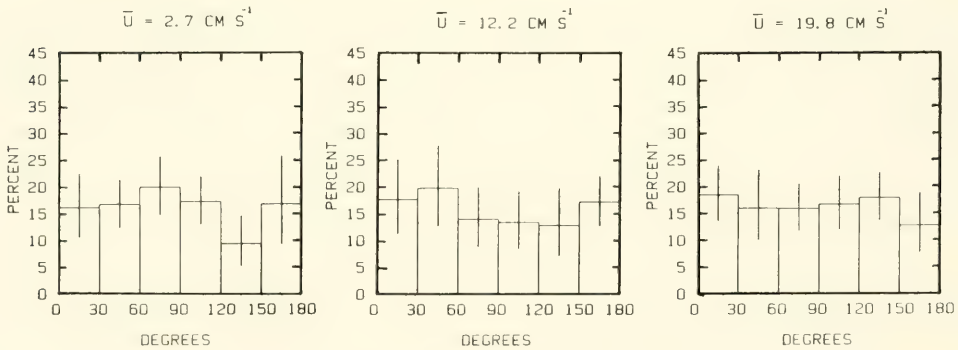


FIGURE 6. Angular position of prey capture events for increased levels of turbulence at three different flow speeds. Data from the bilateral halves of the colony are pooled; hence the abscissa runs from 0 to 180 degrees, with 180 degrees being the extreme downstream point. Capture frequencies are normalized relative to the amount of colony area (and hence number of polyps) available for prey capture. Vertical bars are 95% confidence intervals. For flow speeds of 2.7, 12.2, and 19.8 $cm \cdot s^{-1}$, the number of colonies fed were 10, 10, and 11, and the total number of cysts caught were 383, 305, and 747, respectively.

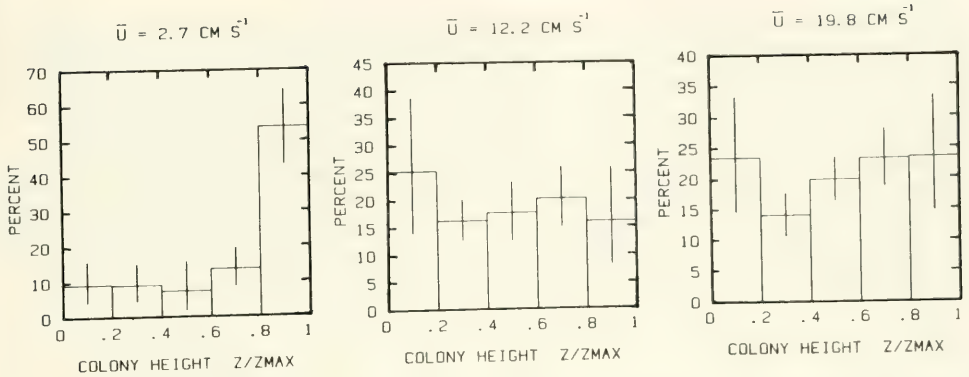


FIGURE 7. Vertical position of prey capture in three flows without turbulence reduction. Z is the distance above the substrate and it has been normalized to ZMAX, the height of the colony. Capture frequencies have been normalized relative to the amount of area avilable for prey capture. Vertical bars are 95% confidence intervals. For flow speeds of 2.7, 12.2, and 19.8 cm·s^{-1}, the number of colonies fed were 11, 10, and 11, and the total number of cysts caught were 346, 342, and 687, respectively.

ronment encountered by passive suspension feeders in such habitats is apt to be quite complicated three-dimensionally. Waves entering the shallow water over these habitats induce oscillating flows which become more important the closer one moves toward any physical boundary in the flow, such as the bottom (Madsen, 1976). Such wave-induced surge can often be of the same order of magnitude as tidally driven currents, when time or ensemble averages of flow speed are made at these sites (Patterson, in prep.). The boundary layer near the bottom is further complicated by the presence of irregularities (sessile organisms, crevices, promontories, sand or mud, and boulders) called 'roughness elements' in the geophysical fluid dynamics literature (Nowell and Church, 1979), which can vary in their density and size distribution and in their effect on the turbulence intensity and character of the flow (Komar, 1976). The effect of distributed roughness elements on shallow water benthic communities has only recently been investigated (Eckman *et al.*, 1981; Nowell *et al.*, 1981; Jumars and Nowell, 1984). Turbulence, bottom shear stress, and water flow encountered by benthic invertebrates can vary a great deal on relatively small spatial scales (Eckman, 1983). The effects of upstream roughness elements are manifold, including the generation of boundary layers within boundary layers, further complicating description of the benthic environment; deeper water studies are attempting to gather empirical measures of roughness elements in the field through photogrammetry and scale models in flumes (Paola, unpub.).

The complications of flows in fully rough depth-limited boundary layers make interpretation of feeding experiments performed in a recirculating flow tank less certain, but it is valuable to compare experiments from the less realistic hydraulically smooth case to those where turbulent mixing may be of importance in understanding how natural flows interact with the passive suspension feeding process.

Explanations for asymmetric capture

The asymmetry of particle capture in *A. siderium* seen at lower and higher smooth flow speeds could arise from several effects (Patterson, 1983): (1) differential concentrations of prey near the surface of the colony may occur along the downstream coordinate. This effect might be expected to show some dependence on flow speed and turbulence level. Experiments with neutrally buoyant glass beads suspended in

laminar flows over flat plates indicate that particle concentrations in the boundary layer can become depleted in the downstream direction (Einav and Lee, 1973), and this effect *decreases* with *increasing* flow speed and turbulence. (2) The polyps and their prey capture surfaces, the tentacles, could experience changing degrees of deformation from flow-induced shear stress on the oral disk and form drag on the columns, which may lead to unfavorable orientations for prey capture at certain flow speeds. (3) The eddying wake on the downstream side of the colony changes in size and shape with changing flow speed and may affect prey capture for downstream polyps. Concentrations of particles in the wake may be different from those in the free stream (Lee and Srinivasan, 1978), and this may affect prey capture.

Particle concentrations in the boundary layer

The first hypothesis is supported by experimental data (Fig. 4, Table I). Concentrations of almost neutrally buoyant brine shrimp cysts in the boundary layer of the colony decrease in the downstream direction at lower speeds. Calculations indicate that predation by upstream polyps can account for only 5% of the depletion observed, leaving hydrodynamic effects as the only mechanism possibly responsible for particle migration.

Observations made through a dissecting microscope at 50X indicate that particles are spinning in the shear field near the tops of the polyps; this rotation may be crucial in the generation of forces responsible for the movement of the particles away from the substrate. Particles that entered the colony below the level of the tentacles meandered downstream between the anthocodial stems, and appeared to be moving away from the surface of the colony the further they progressed downstream. An alternative but not mutually exclusive explanation for the depletion is that flow patterns over and through the polyp-studded surface of the colony aid in movement of particles away from the substrate. Visualization of the flow with filaments of rhodamine dye indicated that flow below the level of the tentacles was slow and laminar and that an abrupt transition occurred above the polyps in the character of the flow, with a steep velocity gradient present (Patterson, in prep.). This effect disappears at higher flow speeds and the availability of prey becomes uniform again (Table I).

The asymmetric capture of particles around the surface of the colony disappeared with increasing flow speed. Particles in flows of the scale considered here are influenced by both inertial and viscous forces (Saffman, 1965). Jørgensen (1982, 1983) has implicated shear-induced particle migration as a mechanism for particle capture in active suspension feeders such as molluscs. Detailed studies of the shear field and particle concentration present within the boundary layer of colonies using laser doppler anemometry and high-speed microcinematography would give good descriptions of particle availability and movements; such a study may help resolve the nature of the forces responsible for particle motions.

The scale of turbulence in the flow tank cannot be greater than the diameter of the channel when the flow straighteners are removed (see Fig. 2); the characteristic length scale of turbulence in the field varies from site to site and can vary over an order of magnitude (Patterson, in prep.). Some of these flows have length scales far removed from that in the flow tank, making application of prey capture patterns seen in the tank to some field flows more problematic. More field measurements of flow in benthic microhabitats are needed to address this issue.

Deformation of filter elements

Deformation of polyps does occur (Fig. 5, Table II) with increasing flow speed, and this helps explain the asymmetric nature of capture in the smooth flow. Due to

the low velocities below the level of the tentacles, polyps deform from shear stress acting on the oral disk (Patterson, unpub. data). Form drag of the anthocodia is likely to be important only at the higher flow speeds. The tentacular crown maintains its shape as the polyp is blown over, with the plane of the oral surface forming an increasing angle to the local direction of flow. The crown collapses into an inverted umbrella-like structure only at the highest flow speeds tested (30–40 cm · s ^{1}). Collapse is probably retarded by the stiffening effect on chevron-like arrays of spicules located at the attachment of the tentacles to the anthocodia. Surfaces of large colonies observed in the field occasionally displayed a cascading effect as the polyps were deformed in strong, unsteady flow (>50 cm · s^{-1}); waves of deformation passed through the columns in a manner similar to wind passing over a wheat field. A tropical octocoral, *Erythropodium caribaeorum,* possesses polyps of roughly the same size as *Alcyonium* and displays a similar behavior (pers. obs.).

Patterns of prey capture at the polyp level can be discerned using the same technique as for whole colony capture (Patterson, in prep.). The mode of prey capture seems to be direct interception (*sensu* Rubenstein and Koehl, 1977; Sebens and Koehl, 1984). The collapse of the upstream polyps changes the nature of the boundary layer flow and allows prey items to be injected below the tentacular canopy. While polyps deformed by the flow can still capture prey, they catch proportionately less as the flow speed increases (Patterson, in prep.).

Wake effects

For Reynolds numbers in the range of 100–10,000, particle entrainment in the recirculating wake of a bluff body can be invoked as a mechanism favoring prey capture in the lee of a suspension feeding organism. Prey capture might be augmented for downstream polyps by the following mechanisms: (1) higher concentrations of particles may occur in the eddying wake than in the free stream, (2) flow speeds over the filter elements may be reduced in the wake, possibly bringing the mean flow speed into some optimum range for the size and shape of the elements (Riedl and Forstner, 1968; Wainwright *et al.,* 1976), and (3) turbulence in the recirculating eddies may allow a greater probability of particle interception from enhanced diffusivity of particles and a more even distribution of particle concentrations immediately adjacent to the polyps.

The first hypothesis is not supported by the data in Table I. Particle concentrations in the wake of colonies are no higher than those in the free stream. This may be understood as follows. The density of planktonic organisms is usually close to that of water. Since the conservation of mass is equivalent to the conservation of volume in an incompressible fluid such as water, the volume of water entering the wake area must be equal to the amount of water leaving the wake. *A priori,* if a particle follows the streamlines of water movement, particle concentrations in the wake can never be higher than freestream values and should be equal. However, large changes in velocity are not accommodated by real particles which, due to their size, slight difference in density, and hence inertia relative to water molecules, deviate from the path of the water. Flow patterns experience sudden changes in speed and direction in and near the boundary of the wake. Vortices are typically shed in a 'von Kármán street' (Vogel, 1981) behind colonies at environmentally realistic flow speeds, and the radius of curvature of water motion in these areas can be on the order of a cm. Particles with sufficient inertia may not be able to make the turn into the wake area near the edge of the wake, and may instead continue downstream. The local pattern of accelerations and decelerations in the wake area may thus determine whether concentrations of particles in the wake will differ from those in the free stream. Unfortunately,

motions of particles at this size range are governed by both viscous and inertial effects (Spielman, 1977), and thus it is difficult to construct analytical models of particle behavior in such flows.

However, these results stand in contrast to laboratory work using laser doppler anemometry which can measure particle concentration with very fine spatial resolution (Bunimovich and Kudin, 1982). This technique has shown that part of the wake of a bluff body may experience an order of magnitude increase in particle concentration over the free stream (Lee and Srinivasan, 1978). The mechanism responsible for this phenomenon is unknown.

Particles dense enough to settle out over the depth of the wake on a time scale comparable to the average 'residence time' of a particle in the wake may be concentrated there (*cf.* gravitational deposition, Rubenstein and Koehl, 1977). This phenomena helps explain why rear windows on cars get dirtier than windshields (T. A. McMahon, Harvard University, pers. comm.), but may be of limited relevance to marine suspension feeders which usually consume prey with longer settling times (minutes) than typical wake residence times (seconds).

Direct interception is inversely dependent on flow velocity (Spielman, 1977; LaBarbera, 1984), but post-interception shear stresses on captured particles are often strong enough to dislodge them. The strength of attachment of nematocyst penetrants and threads is an unstudied area. The capture data reported here include successful captures only; J. Miles (Northeastern University, pers. comm.) has found that the loss of particles shows an increase with flow speed in the sea anemone *Metridium senile.* If a similar phenomenon occurs in *Alcyonium,* then the shift in capture may result from a shift in the location of an optimal local flow speed over the polyps. One might then expect the Reynolds number calculated for the most successful polyps in each set of experiments to be about the same, but this was not the case. Okamura (University of California, Berkeley, pers. comm.) has suggested that distributions of prey capture seen in the arborescent bryozoan *Bugula* may be due to a greater availability of particles to downstream branches as the flow speed increases. Upstream branches become increasingly less able to capture prey which then makes it further downstream. Also, 'conditioning' of the flow may also be important in this case, similar to that occurring in water movement through the alga *Gelidium* (Anderson and Charters, 1982). Upstream branches slow the mean flow speed while increasing levels of turbulence which can promote capture from turbulent diffusion.

While mean flow speeds are markedly lower in the wake, the level of turbulence increases (Schlicting, 1968); particles passing over the polyps in the wake have a higher probability of encountering a tentacle if momentum is being exchanged between layers of water near the colony. Turbulence enhances diffusivity of both motile and non-motile particles (Bird *et al.,* 1960). Hence, even if particle concentrations are lower in the wake at the highest speeds tested, turbulence will aid in prey capture. The disappearance of a downstream depletion of particles in the boundary layer of the colony due to migratory effects also probably results from the boundary layer becoming turbulent as the Reynolds number increases.

When the turbulence is increased in the free stream by moving the flow straighteners, the flow more closely resembles that found on the subtidal rock walls, and the effect disappears. Angular position on the colony is no longer a factor in predicting feeding success, for all polyps have an equal probability in capturing prey. Deformation still occurs, but the downstream gradient in particle concentration in the boundary layer of the colony does not occur. This seems to indicate that the depletion effect is the major factor governing distributions in the experiments with flow straighteners.

In the experiments with increased levels of turbulence, polyps further away from the substrate captured more prey only at the lowest speeds tested. This may underly

the observation that young colonies grow away from the substrate faster than they expand laterally. If the assumption is made that those polyps which eat more food have more polyps emerging adjacent to them from inside the colony, colonies should exhibit this growth pattern. In stagnant areas, colonies are taller and thinner relative to colonies in more well-mixed areas (Patterson, 1980). At higher flow speeds, the distribution again becomes asymmetric, this time with polyps at both the bottom and top of the colony catching relatively more prey. Since colonies of different heights displayed this bimodality, it seems likely that the pattern reflects the horseshoe vortex which forms on the upstream side of obstacles in flow near a substrate (Schlicting, 1968).

The observations above raise the interesting question of whether colony shape can be predicted from knowledge of whole colony capture patterns and *vice versa*. *Alcyonium* colonies display a variety of shapes including spherical geometries in hydraulically rough areas, and ellipsoids oriented at right angles to flow in smoother habitats. One might suspect that in a microhabitat where the direction of flow is predictable, colonies should grow fastest at the point where the flow speed around the colony is the greatest, at the 90° positions. Such a colony would eventually have an ellipsoidal shape. Colonies in a microhabitat subject to changing current direction would capture food in all directions and thus obtain a spherical geometry with time. However, these suspicions are technically difficult to test given the slow growth rate of the young colonies (Sebens, 1983c) and the difficulties of long-term monitoring and characterization of flow.

The mechanics of prey particle motion in the boundary layers of passive suspension feeders and the location and deformation of filter elements is of crucial importance to the feeding process. In the soft coral *Alcyonium*, the character of the flow carrying food past the filters can dramatically change the pattern of whole colony capture. Upon close inspection, many other passive suspension feeders may operate in a similar manner.

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LITERATURE CITED

ABBOTT, B. M. 1974. Flume studies in the stability of model corals as an aid to quantitative paleoecology. *Paleogeogr. Paleoclimatol. Paleoecolog.* **15:** 1–27.

ANDERSON, S. M., AND A. C. CHARTERS. 1982. A fluid dynamics study of seawater flow through *Gelidium nudifrons*. *Limnol. Oceanogr.* **27:** 399–412.

BIRD, R. B., W. E. STEWART, AND E. N. LIGHTFOOT. 1960. *Transport Phenomena*. John Wiley, New York. 780 pp.

BRADSHAW, P. 1971. *An Introduction to Turbulence and its Measurement*. Pergamon Press, New York. 218 pp.

BUNIMOVICH, L. A., AND A. M. KUDIN. 1982. Determination of concentrations of marine suspensions by means of a laser anemometer. *Oceanology* **22:** 498–500.

CHAMBERLAIN, J. A., JR., AND R. R. GRAUS. 1975. Water flow and hydromechanical adaptations of branched reef corals. *Bull. Mar. Sci.* **25:** 112–125.

ECKMAN, J. E. 1983. Hydrodynamic processes affecting benthic recruitment. *Limnol. Oceanogr.* **28:** 241–257.

ECKMAN, J. E., A. R. NOWELL, AND P. A. JUMARS. 1981. Sediment destabilization by animal tubes. *J. Mar. Res.* **39:** 361–374.

EINAV, S., AND S.-L. LEE. 1973. Particles migration in laminar boundary layer flow. *Int. J. Multiphase Flow* **1:** 73–88.

FELDMAN, S. L. 1976. Validation of the species of soft coral *Alcyonium siderium:* description and reproductive biology. M. Sc. thesis, Northeastern University.

GRIGG, R. W. 1972. Orientation and growth form of sea fans. *Limnol. Oceanogr.* **17:** 185–192.

HEWITT, G. F. 1978. *Measurement of Two Phase Flow Parameters.* Academic Press, New York. 287 pp.

JOHANNES, R. E., S. L. COLES, AND N. T. KUENZEL. 1970. The role of zooplankton in the nutrition of some scleractinian corals. *Limnol. Oceanogr.* **15:** 579–586.

JØRGENSEN, C. B. 1949. The rate of feeding by *Mytilus* in different kinds of suspension. *J. Mar. Biol. Assoc. U.K.* **28:** 333–344.

JØRGENSEN, C. B. 1955. Quantitative aspects of filter feeding in invertebrates. *Biol. Rev.* **30:** 391–454.

JØRGENSEN, C. B. 1982. A hydromechanical principle for particle retention in *Mytilus edulis* and other ciliary suspension feeders. *Mar. Biol.* **61:** 277–282.

JØRGENSEN, C. B. 1983. Fluid mechanical aspects of suspension feeding. *Mar. Ecol. Prog. Ser.* **11:** 89–103.

JUMARS, P. A., AND A. R. M. NOWELL. 1984. Fluid and sediment dynamic effects on marine benthic community structure. *Am. Zool.* **24:** 45–55.

KOEHL, M. A. R. 1977. Water flow and the morphology of zoanthid colonies. *Proc. 3rd Int. Coral Reef Symp., Miami* **1:** 437–444.

KOMAR, P. D. 1976. Boundary layer flow under steady unidirectional currents. Pp. 91–106 in *Marine Sediment Transport and Environmental Management,* D. J. Stanley and D. J. P. Swift, eds. John Wiley, New York.

LABARBERA, M. 1978. Particle capture by a Pacific brittle star: experimental test of the aerosol suspension feeding model. *Science* **201:** 1147–1149.

LABARBERA, M. 1981. Water flow patterns in and around three species of articulate brachiopods. *J. Exp. Mar. Biol. Ecol.* **55:** 185–206.

LABARBERA, M. 1984. Feeding currents and particle capture mechanisms in suspension feeding animals. *Am. Zool.* **24:** 71–84.

LABARBERA, M., AND S. VOGEL. 1976. An inexpensive thermistor flowmeter for aquatic biology. *Limnol. Oceanogr.* **21:** 750–756.

LASKER, H. R. 1981. A comparison of the particulate feeding abilities of three species of gorgonian soft coral. *Mar. Ecol. Prog. Ser.* **5:** 61–67.

LEE, S.-L., AND J. SRINIVASAN. 1978. Measurement of local size and velocity probability density distributions in two-phase suspension flows by laser-doppler technique. *Int. J. Multiphase Flow* **4:** 141–155.

LEVERSEE, G. J. 1976. Flow and feeding in fan-shaped colonies of a gorgonian coral, *Leptogorgia. Biol. Bull.* **151:** 344–356.

MACURDA, D. B., AND D. L. MEYER. 1974. Feeding and posture of modern stalked crinoids. *Nature* **247:** 394–396.

MADSEN, O. S. 1976. Wave climate of the continental margin: elements of its mathematical description. Pp. 65–90 in *Marine Sediment Transport and Environmental Management,* D. J. Stanley and D. J. P. Swift, eds. John Wiley, New York.

MEYER, D. L. 1973. Feeding behaviour and ecology of shallow-water unstalked crinoids (Echinodermata) in the Caribbean Sea. *Mar. Biol.* **22:** 105–129.

MUSCATINE, L., AND J. W. PORTER. 1977. Reef corals: mutualistic symbioses adapted to nutrient poor environments. *BioScience* **27:** 454–460.

NOWELL, A. R., AND M. A. CHURCH. 1979. Turbulent flow in a depth-limited boundary layer. *J. Geophys. Res.* **84:** 4816–4824.

NOWELL, A. R., P. A. JUMARS, AND J. E. ECKMAN. 1981. Effects of biological activity on the entrainment of marine sediments. *Mar. Geol.* **42:** 133–154.

O'NEILL, P. L. 1978. Hydrodynamic analysis of feeding in sand dollars. *Oecologia* **34:** 157–174.

PARKER, G. J. 1968. Some factors governing the design of probes for sampling in particle and drop-laden streams. *Atmos. Environment* **2:** 477–490.

PATTERSON, M. R. 1980. Hydromechanical adaptations in *Alcyonium siderium* (Octocorallia). Pp. 183–201 in *Biofluid Mechanics 2,* D. J. Schneck, ed., Plenum, New York.

PATTERSON, M. R. 1983. Asymmetrical particle capture in a marine filter feeder. *J. Biomechanics* **16:** 279. (Abstract)

PORTER, J. 1974. Zooplankton feeding by the Caribbean reef-building coral *Montastrea cavernosa. Proc. 2nd Int. Coral Reef Symp.* **1:** 111–125.

PORTER, J. 1979. Adaptive geometry of zooplankton feeding in reef corals. (Abstract). *42nd Ann. Meeting Am. Soc. Limnol. Oceanogr.*

PURCELL, J. E. 1977. The diet of large and small individuals of the sea anemone *Metridium senile*. *Bull. S. Cal. Acad. Sci.* **76:** 168–172.

REES, J. T. 1972. The effect of current on the growth form in an octocoral. *J. Exp. Mar. Biol. Ecol.* **10:** 115–124.

RIEDL, R. J. 1971. Water movement. Pp. 1085–1088, 1124–1156 in *Marine Ecology, Vol. 1, Part 2,* O. Kinne, ed. Wiley Interscience, London.

RIEDL, R. J., AND H. FORSTNER. 1968. Wasserbewegung im Mikrobereich des Benthos. *Sarsia* **34:** 163–168.

ROBBINS, K. E., AND J. M. SHICK. 1980. Expansion-contraction behavior in the sea anemone *Metridium senile:* environmental cues and energetic consequences. Pp. 101–106 in *Nutrition in the Lower Metazoa,* D. C. Smith and Y. Tiffon, eds. Pergamon Press, New York.

RUBENSTEIN, D. J., AND M. A. R. KOEHL. 1977. The mechanism of filter feeding: some theoretical considerations. *Am. Nat.* **111:** 981–994.

SAFFMAN, P. G. 1965. The lift on a small sphere in a slow shear flow. *J. Fluid Mech.* **22:** 385–400.

SCHLICTING, H. 1968. *Boundary Layer Theory.* McGraw-Hill, New York. 747 pp.

SEBENS, K. P. 1977. Autotrophic and heterotrophic nutrition of coral reef zoanthids. *Proc. 3rd Int. Coral Reef Symp. Miami* **1:** 397–404.

SEBENS, K. P. 1981. The allometry of feeding, energetics, and body size in three sea anemone species. *Biol. Bull.* **161:** 152–171.

SEBENS, K. P. 1982. Competition for space: prey availability and escape in size. *Am. Nat.* **120:** 189–197.

SEBENS, K. P. 1983a. Larval and juvenile ecology of the temperate octocoral *Alcyonium siderium* Verrill. I. Substratum selection by benthic larvae. *J. Exp. Mar. Biol. Ecol.* **71:** 73–89.

SEBENS, K. P. 1983b. Settlement and metamorphosis of temperate soft-coral larva (*Alcyonium siderium* Verrill): induction by crustose algae. *Biol. Bull.* **165:** 286–304.

SEBENS, K. P. 1983c. Larval and juvenile ecology of the temperate octocoral *Alcyonium siderium* Verrill. II. Fecundity, survivorship, and juvenile growth. *J. Exp. Mar. Biol. Ecol.* **72:** 263–285.

SEBENS, K. P. 1984. Water flow and coral colony size: interhabitat comparisons of the octocoral *Alcyonium siderium. Proc. Natl. Acad. Sci.* **81:** 5473–5477.

SEBENS, K. P., AND M. A. R. KOEHL. 1984. Predation on zooplankton by the benthic anthozoans, *Alcyonium siderium* (Alcyonacea) and *Metridium senile* (Actiniaria) in the New England subtidal. *Mar. Biol.* **81:** 255–271.

SPIELMAN, L. A. 1977. Particle capture from low speed laminar flows. *Ann. Rev. Fluid Mech.* **9:** 297–319.

SVOBODA, A. 1976. The orientation of *Aglaophenia* fans to current in laboratory conditions (Hydrozoa, Coelenterata). Pp. 41–48 in *Coelenterate Ecology and Behavior,* G. O. Mackie, ed. Plenum, New York.

TELFORD, M. 1981. A hydromechanic interpretation of sand dollar morphology. *Bull. Mar. Sci.* **31:** 605–622.

THÉODOR, J., AND DENIZOT. 1965. Contribution a l'étude des Gorgones III. Trois formes adaptives d'*Eunicella stricta* en fonction de la turbulence et du courant. *Vie Milieu* **14:** 815–818.

VOGEL, S. 1981. *Life in Moving Fluids: the Physical Biology of Flow.* Willard Grant Press, Boston. 352 pp.

VOGEL, S., AND M. LABARBERA. 1978. Simple flow tanks for research and teaching. *BioScience* **10:** 638–643.

VOSBURGH, F. 1977. The response to drag of the reef coral *Acropora reticulata. Proc. 3rd Int. Coral Reef Symp. Miami* **1:** 477–482.

WAINWRIGHT, S. A., AND M. A. R. KOEHL. 1976. The nature of flow and reaction of benthic cnidaria to it. Pp 5–21 in *Coelenterate Ecology and Behavior,* G. O. Mackie, ed. Plenum, New York.

WAINWRIGHT, S. A., W. D. BIGGS, J. D. CURREY, AND J. M. GOSLINE. 1976. *Mechanical Design in Organisms.* John Wiley, New York. 423 pp.

WARNER, G. F. 1971. On the ecology of a dense bed of the brittle-star *Ophiothrix fragilis. J. Mar. Biol. Assoc. U.K.* **51:** 267–282.E

WARNER, G. F. 1976. On the shapes of passive suspension feeders. Pp. 567–575 in *Biology of Benthic Organisms,* B. F. Keegan, P. O. Ceidigh, and P. J. S. Boaden, eds. Pergamon Press, New York.

WARNER, G. F., AND J. D. WOODLEY. 1975. Suspension feeding in the brittlestar *Ophiothrix fragilis. J. Mar. Biol. Assoc. U.K.* **55:** 199–210.

ZAR, J. H. 1974. *Biostatistical Analysis.* Prentice-Hall, Englewood Cliffs, New Jersey. 620 pp.

Reference: *Biol. Bull.* **167**: 630–646. (December, 1984)

OBSERVATIONS ON THE LONG-TERM POPULATION DYNAMICS OF THE PERENNIAL ASCIDIAN, *ASCIDIA MENTULA* O. F. MÜLLER, ON THE SWEDISH WEST COAST

IB SVANE

Kristineberg Marine Biological Station, Kristineberg 2130, S-450 34 Fiskebäckskil, Sweden

ABSTRACT

Six populations of *Ascidia mentula* O. F. Müller on subtidal vertical rock walls were monitored continually for 12 years (1971–1982) using stereophotographic techniques. Three stations at two depth levels were observed, along a hydrographical gradient extending 100 km from the sheltered inner parts of the Gullmarsfjord to the exposed archipelago off the Swedish west coast. Population densities increased during 1971–1976, and gradually declined from 1976. Recruitment was density-dependent while mortality was density-independent. Temporal covariation in recruitment between stations and depths separated fjord stations into two independent, correlated patterns: (1) the exposed archipelago station and the shallow semi-sheltered fjord station from (2) the shallow-sheltered fjord station from the sheltered and deep semi-sheltered fjord station. Temporal covariation in mortality separated the exposed station from the sheltered fjord stations suggesting different mortality factors. Histological analysis of gonads and analysis of photographically monitored recruitment revealed a seasonal reproductive pattern at 15 m depth while continuous reproduction and recruitment was observed in deeper populations. Temporal patterns of population density appeared to be related to long-term hydrographic changes mediated by variation in recruitment. Mortality caused by predation was not observed but disturbance and dislocation by sea urchins was an important mortality factor, especially at exposed sites.

INTRODUCTION

Study of fouling communities on submerged artificial substrates has contributed much to our knowledge of the structure and dynamics of subtidal epifaunal communities. However, the physical properties and the emplacement and subsequent handling of artificial substrates makes close comparison with natural substrates somewhat difficult (Dean, 1981; Kay and Keough, 1981; Schoener and Greene, 1981; Field, 1982). The development and regulation of communities established on artificial substrates have been of prime interest for investigators interested in such interactions as predation, competition and successional patterns (*e.g.,* Osman, 1977; Sutherland and Karlson, 1977; Karlson, 1978; Sutherland, 1981; Chalmer, 1982). These and other studies in the rocky intertidal (*e.g.,* Dayton, 1971; Menge, 1976) recognized the importance of seasonal and temporal variations in recruitment but, due to the relatively short term observations, inter-annual variation in recruitment have not been incorporated into explanations of the community structure.

The fauna of the rocky west coast of Sweden is greatly influenced by the Baltic current. This current is observed in the stratified water column as the upper, low salinity layer above a deeper, oceanic layer. Below the halocline the epifauna on the

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granite rock walls is dominated by solitary ascidians, brachiopods, and tube-building polychaetes. Here annual ascidians temporarily reach high densities while perennial species show less pronounced fluctuations in density (Svane, 1983). *Ascidia mentula* O. F. Müller is a quantitatively important, perennial species found exclusively on hard substrates. It can reach high densities at depths greater than 15 m. It is rarely found above the 15 m level. *A. mentula* has a boreal-lusitanian distribution (Berrill, 1950) and is recorded as far north as the Trondheimsfjord on the west coast of Norway (Millar, 1966).

A detailed study of growth, reproduction, and long-term population dynamics of *A. mentula* at one station and depth is described by Svane and Lundälv (1981). The aim of the present study is to assess the role of reproduction, recruitment, and mortality in the spatial and temporal population dynamics of *A. mentula*. An attempt has been made to correlate changes in physical environmental parameters with patterns of recruitment, reproduction and mortality observed at three stations each at two depth levels over twelve consecutive years.

Material and Methods

A stereo-photogrammetrical analysis was undertaken on data obtained over a 12-year period (1971–1982) from three stations, each at two depth levels, on the Swedish west coast (Fig. 1). Station S1 is at an exposed locality (St. Sundskär 58°32.7′N, 11°3.3′E) in the Väderö archipelago off the Swedish west coast. Station G6 is in a

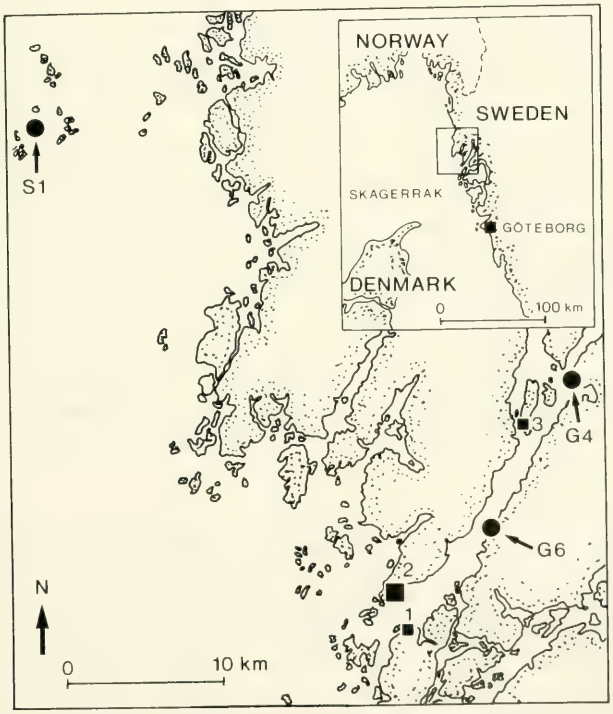


FIGURE 1. Map showing the Skagerrak area (inset) and the stations: S1: the exposed station in the Väderö archipelago. G6 and G4: the semisheltered and sheltered stations in the inner and central part of the Gullmarsfjord. 1. Kristineberg Marine Biological Station. 2. The town of Lysekil. 3. Bornö Hydrographical Station.

semi-sheltered locality (Gåsklåvan 58°23.7′N, 11°32.4′E) situated in the central part of the Gullmarsfjord. Station G4 is in a sheltered locality (Smörkullen 58°23.7′N, 11°37.9′E) situated in the inner part of Gullmarsfjorden. All stations are nearly vertical granite rock walls. Station S1 faces SSE and G6 and G4 face NW and W, respectively. The two depth levels at stations G4 and G6 were 15 m and 20 m; at station S1 they wee 20 m and 25 m. Observations of the 25 m site at station G6 and the 15 m site at station S1 do exist but *A. mentula* is not very common on the photographed areas. At station G4 the 20 m level is the deepest site as the fjord bottom is at 25 m depth. The rock walls at each site had the following mean inclination: S1, 20 m: 85°; S1, 25 m: 96°; G6, 15 m: 90°; G6, 20 m: 97°; G4, 15 m: 110°; G4, 20 m: 113°. At each station and depth level six test squares (a total area of 1.5 m^2) were inspected by SCUBA-diving and stereophotographed at regular intervals 3–7 times each year. The underwater photogrammetrical method and its limitations have been described by Lundälv (1971) and Torlegård and Lundälv (1974).

The population density, recruitment, and mortality figures were obtained by following individual animals throughout their observable benthic stages. Settling times and death, were estimated by interpolation between sampling dates and set arbitrarily at the middle of the period between two observations. Newly settled animals could be observed at a size of 2–3 mm, thus settling larvae and early postlarvae were not observed. Recruitment and mortality rates were calculated as follows: $r_{M,R} = \frac{X_{t_0-t_1}}{N_{t_0} \cdot \Delta t}$ where $X_{t_0-t_1}$ is the number which appeared or disappeared during the interval, N_{t_0} is the density at the start of the interval, and Δt is the time interval. Mortality rates and recruitment, calculated in one month intervals at the six locations, were tested for temporal covariation by correlation analysis (Sokal and Rohlf, 1981) and the correlation coefficients were clustered according to Mountford's (1962) method. Live material, for the study of reproduction and gonad histology, was obtained by regular diving during two periods and at two locations: during 1979 at Hågarnskär in the mouth of the fjord at 25 m depth as described by Svane and Lundälv (1981), and during 1980–1982 in the vicinity of station G6, in the central part of the fjord. Samples were obtained about every three weeks at station G6 at 15 m depth as in the 1979 study. The animals were fixed in Bouin's fluid and later transferred to 70% ethanol. The gonads were dissected out, embedded in Paraplast, cut into 8 μm sections, and stained in hematoxylin-eosin. The diameters of oocytes in one slide, all with the nucleolus showing in section, were measured for each ovary sample and the size frequency distribution in percent of total numbers was determined (see Giese and Pearse, 1974). Oocytes with a diameter above 125 μm were considered mature since no difference in staining or morphology was apparent in this size group.

Hydrographical properties of the study sites

Daily records of temperatures and salinities were obtained from Bornö Hydrographical Station (National Board of Fisheries, Sweden) for 15 and 20 m depths over the studied period. The recording station is located in the inner part of the Gullmarsfjord close to stations G4 and G6 (Fig. 1). Additional records (4–12 times per year, 1972–1977), were reported in Svane and Lundälv (1981) from Lysekil archipelago. Monthly mean temperatures and salinities are shown in Figure 2. Deviations from the monthly means were calculated at both depth levels and also depicted in Figure 2.

The study sites are influenced by two water masses, usually separated by a distinct halocline and often by a thermocline as well. The upper water mass is the so-called

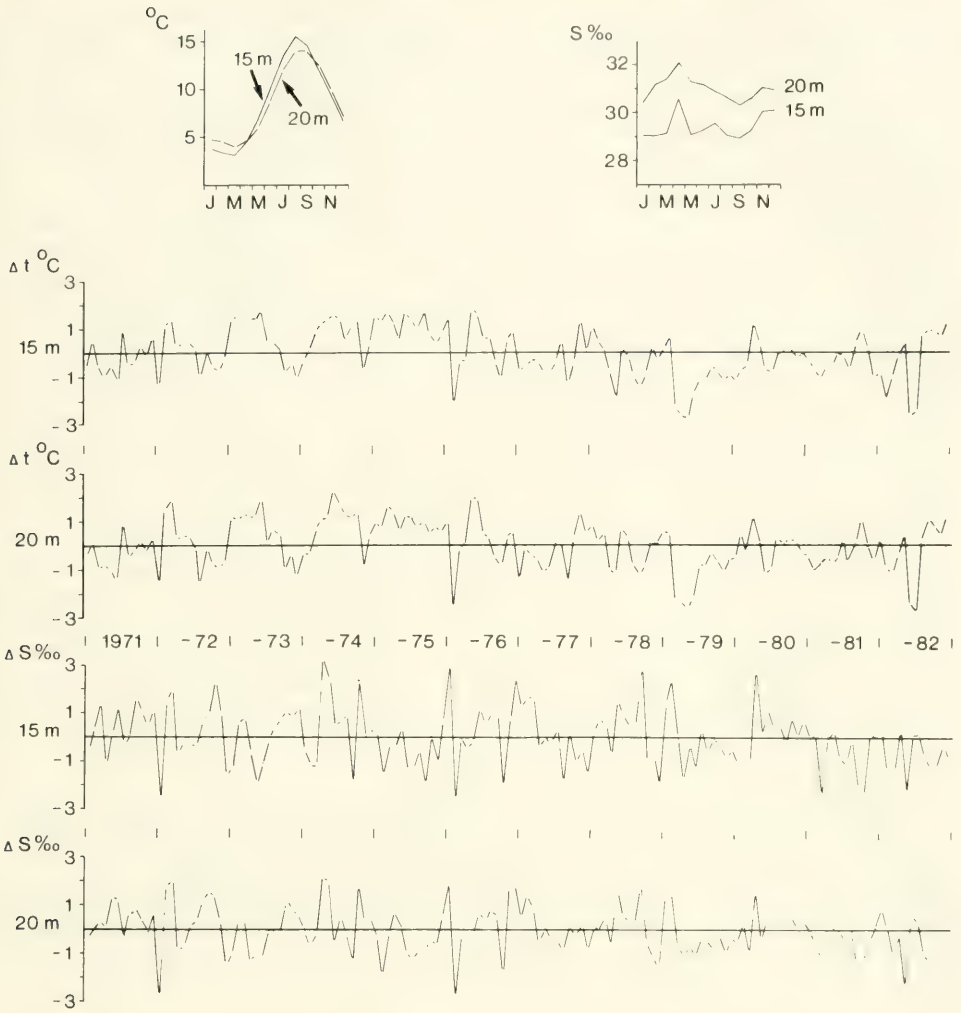


FIGURE 2. Below: temperature and salinity anomalies at two depth levels (15 and 20 m) calculated as the mean monthly deviation from the 12-year mean. Above: mean yearly temperature (left) and salinity (right) patterns at two depth levels. Data from Bornö Hydrographical Station in the inner part of the Gullmarsfjord.

"Baltic-water," influenced by outflow from the Baltic Sea (S < 30‰). The lower water mass (S = 31–34‰) originates in the Skagerrak and is derived ultimately from the open North Sea. The discontinuity between these two water masses is normally found at depths between 10 and 15 m but is subject to rapid vertical displacements of considerable magnitude and may penetrate to a depth of 20 m (see Svansson, 1975).

Faunal components of the study sites

All the stations are dominated by ascidians. Other organisms, however, are constituents of the substratum on which the ascidian populations fluctuate.

On the exposed station S1 background organisms are mainly the tube building polychaete, *Pomatoceros triqueter* (L.), and the encrusting algae, *Lithothamnion* spp.,

which may cover the monitored areas extensively. Ascidians settle on and among these organisms. The annual ascidians are *Ascidiella scabra* (O. F. Müller), *Ascidiella aspersa* (O. F. Müller), *Corella paralellogramma* (O. F. Müller), and occasionally *Ciona intestinalis* (L.). The perennial ascidians include *Boltenia echinata* (L.) in addition to *A. mentula* as described by Svane and Lundälv (1981, 1982a) and Svane (1983). The sea urchins *Echinus esculentus* (L.) and *Echinus acutus* (Lamarck) are occasionally found and may leave tracks of ascidian-cleared substratum.

The sheltered stations in the fjord have a different composition and a more dense cover of epifaunal organisms. At the 20 m depth levels tube-building polychaetes dominate on the primary substratum. These are principally *Hydroides norvegica* (Gunnerus), *Serpula vermicularis* (L.), and *Chaetopterus variopedatus* (Reinier), and among these tubes the brachiopod *Crania anomala* (O. F. Müller). Patches of *Lithothamnion* spp. are also found, but considerably less than at the exposed sites. Together with *A. mentula* the relatively small perennial ascidians *Pyura tessellata* Forbes and *B. echinata* are fairly stable components at 20 m and deeper compared with the annual ascidians, as described by Svane and Lundälv (1982a, b). Within the fjord the annual ascidian *C. intestinalis* is abundant and shows large fluctuations in density. The small anthozoan *Protanthea simplex* Carlgren is common at both depths. At 15 m the cover of tube-building polychaetes is considerably less and brachiopods are only rarely found. At this depth patches of unidentified bluegreen algae occur and the small ascidian *Dendrodoa grossularia* (Van Beneden) is abundant. Sponges, mainly *Halicondria panicea* (Pallas) and *Haliclona* spp., are also found at both depths, but more commonly at the 15 m levels. Large sea urchins and carnivorous echinoderms are only rarely found, but the starfish *Asterias rubens* L. can be abundant, especially when *Ciona* populations are large (see Svane and Lundälv, 1982a; Svane, 1983).

Results

Throughout the 12-year period of investigation the population densities varied in a similar fashion both among and within stations (Fig. 3). After initial low population densities in 1971, all increased to a maximum in the years 1975–1977, with the exception of station G4 at the 20 m depth which reached its lowest level in 1974, but thereafter followed the group pattern. After 1976–1977 all populations declined except for temporary increases in 1979 at station S1 and in 1980 at stations G6 and G4.

Spatial patterns

Recruitment. The number of recruits varied considerably both among and within stations. The yearly mean density and recruitment for each station and depth were calculated for the entire period of study (Fig. 4A) and showed a positive correlation ($r = 0.93$). A gradient of recruitment intensity at each depth level seems to exist with highest intensity at the exposed station S1 and successive reduction toward the inner part of the fjord. The positive correlation implies that larger populations receive proportionately more recruits. By expressing the recruitment rate as a function of population density (yearly mean values from the entire period of study), it is seen in Figure 4B that maximum recruitment rate was found at a population density of about 100 ind/m^2 above and below which the recruitment rate declines. Station G6, 15 m and S1, 25 m, showed reduced recruitment rates and may have reached their saturation levels. Station G6 and G4 at 20 m, however, were below their saturation levels.

Mortality. The mortality rate (yearly mean values from the entire period of study) was independent of density (Fig. 4C). Highest mortality rates were observed at the

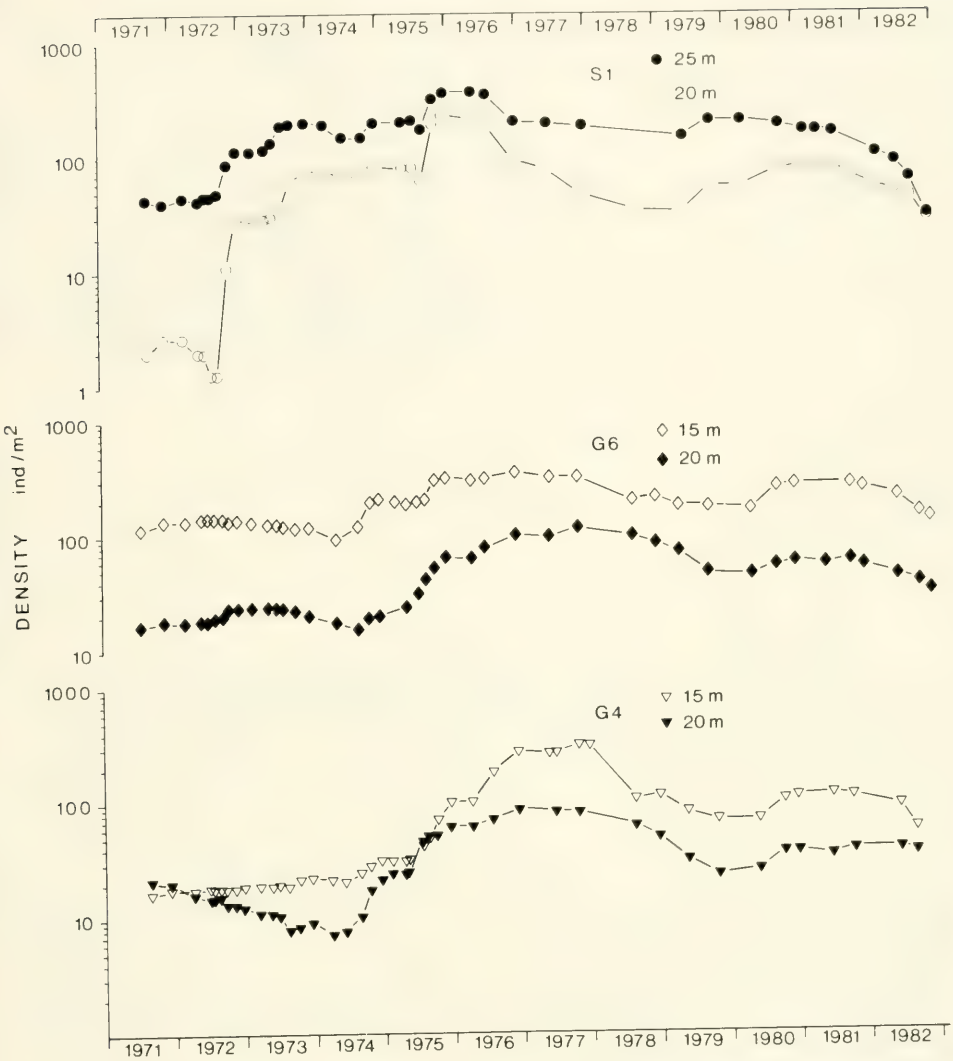


FIGURE 3. Densities of six populations of *Ascidia mentula* at three stations each at two depth levels (log scale). S1: the exposed station in the Väderö archipelago off the Swedish west coast. G6 and G4: the semi-sheltered and sheltered stations in the central and inner part of the Gullmarsfjord.

exposed station S1 (1 and 2) and lowest at the intermediate station G6 (3 and 4). The mean mortality rate was 40 ind/m^2/year and recruitment consequently balanced mortality at two levels of density. A single individual at station G6, 15 m reached an age of 10.5 years, but maximum age at the remaining stations was about 8 years. However, the majority of individuals survived about two years.

No substantial predation was observed and dying animals were observed over long periods through lack of growth, shrinkage, and color-changing (see Svane and Lundälv, 1981). Mortality caused by disturbance and dislocation by sea urchins was important, especially at the exposed sites. Here browsing sea urchins were observed to "bulldoze" over the substratum and leave cleared tracks. When *A. mentula* was found in dense aggregates sea urchin activity was to some extent prevented since

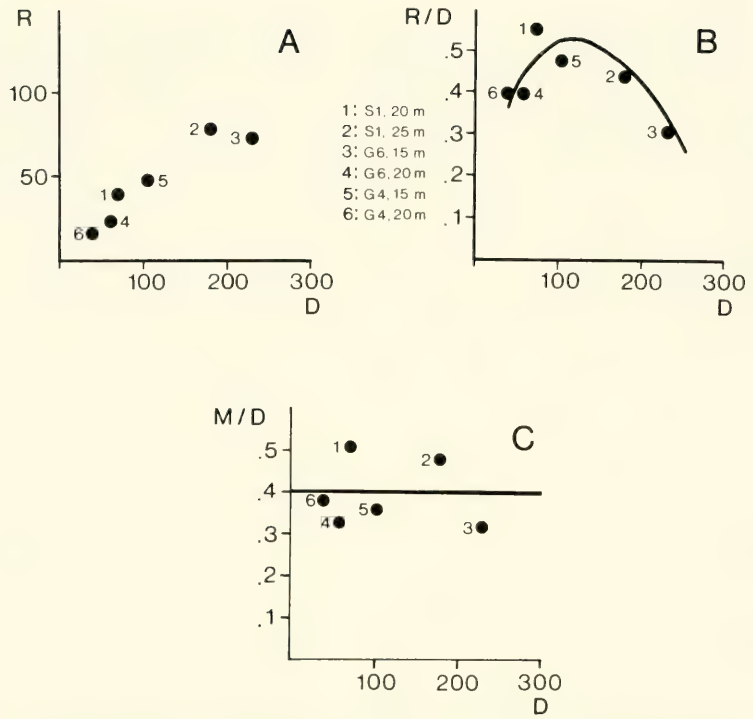


FIGURE 4. Recruitment (recruits/m^2/year) (A) and recruitment rate (recruits/ind.) (B) and mortality rate (dead ind./ind.) (C) as a function of density (12-year mean values; each station and depth shown separately).

aggregates seemed to act as a rampart. Occasionally sea urchins carrying spine-stabbed ascidians were observed. At the sheltered sites large sea urchins were not common and relatively dense epifaunal cover restricted their movements and "bulldozing" effect.

Reproduction. Svane and Lundälv (1981) reported that *A. mentula* reproduced throughout the year with maximum intensity in October-November. Evidence was based on analysis of live material sampled at 25 m depth close to the mouth of the fjord (Fig. 5A). Later sampling performed at the 15 m level in the vicinity of station G6 in the central part of the fjord, revealed a seasonal pattern in oocyte size-frequency (Fig. 5B). At the 15 m depth level mature oocytes were only found from July to December with some seasonal differences between the two years studied, while at the 25 m level mature oocytes were found throughout the year. In animals sampled at 15 m, sperm were occasionally found in the gonoducts during the winter and spring (January–May), but no oocytes. At 25 m more than 50% of the animals carried oocytes or sperm (Svane and Lundälv, 1981). By summarizing monthly recruitment from the 12-year period of study the relative recruitment at each station and depth level was determined (Fig. 6). At the 15 m level (G6 and G4) very little recruitment took place during the winter months of January, February, and March compared to the other locations. Maximum intensity of recruitment occurred in September-October with the exception of station G4, 20 m, where maximum intensity occurred in July-August. At station G4, 20 m, recruitment was generally poor (Fig. 7).

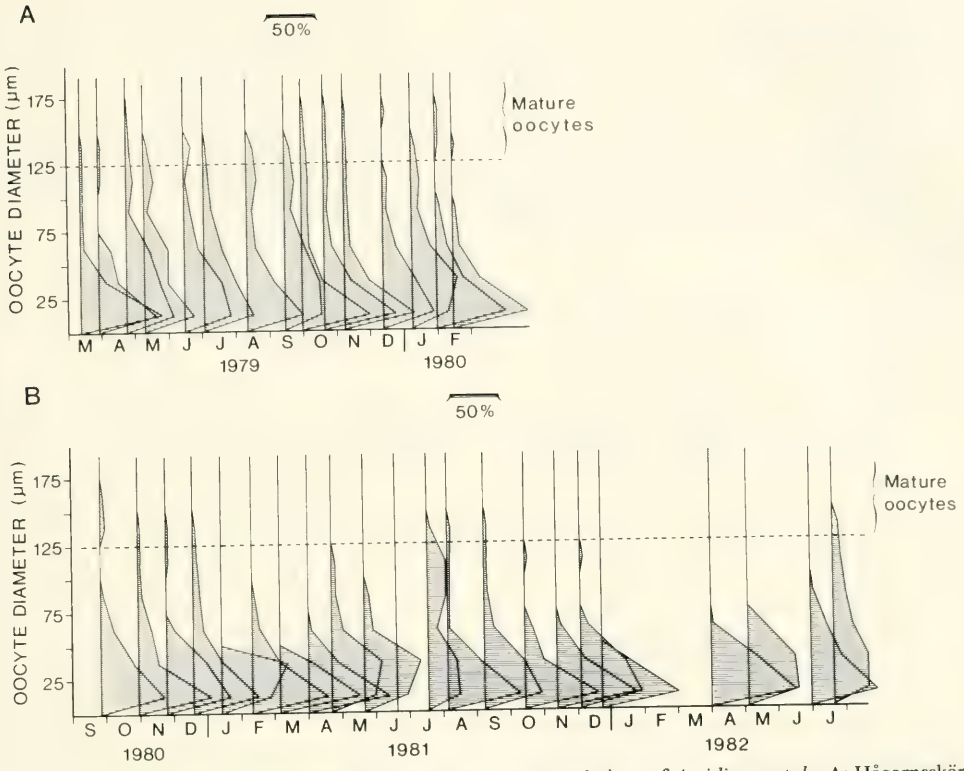


FIGURE 5. Oocyte size-frequency diagrams from two populations of *Ascidia mentula*. A: Hågarnsskär in the mouth of the Gullmarsfjord at 25 m depth. B: Gåsklåvan (the vicinity of station G6) in the central part of Gullmarsfjorden at 15 m depth. (Scale indicates 50% of total numbers in each sample).

Temporal patterns

The density patterns throughout the 12 years were similar although the stations are separated geographically by several kilometers (Fig. 1). However, recruitment and mortality factors were operating differently at different stations and depths. The overall

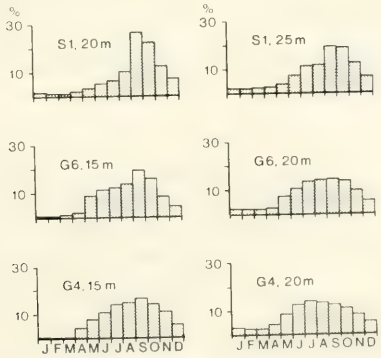


FIGURE 6. The mean recruitment at three stations each at two depth levels summarized from a 12-year period and calculated in one-month intervals.

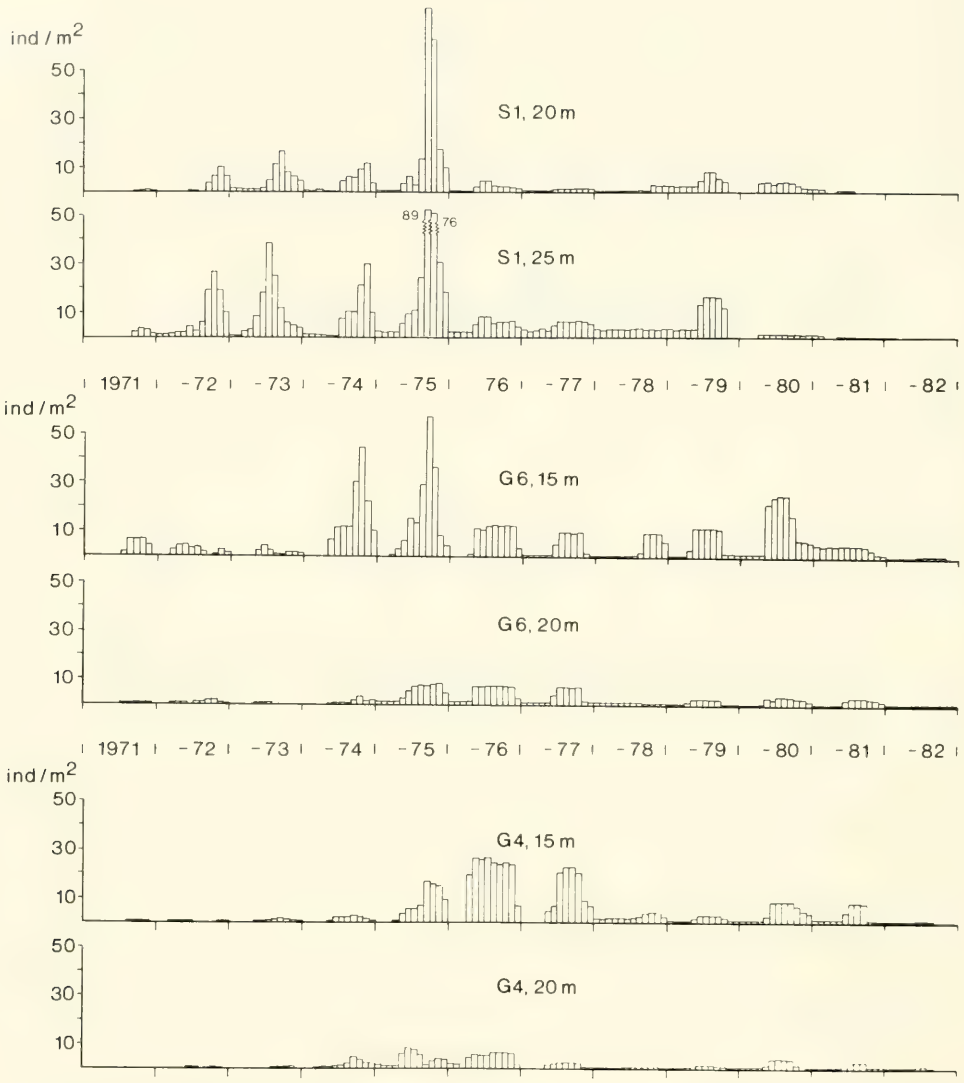


FIGURE 7. Recruitment in six populations of *Ascidia mentula* at three stations each at two depth levels calculated in one-month intervals.

recruitment is depicted in Figure 7, showing the number of recruits observed at different stations and depths throughout the 12-year period of study, calculated in 1-month intervals. The overall mortality rates are depicted in Figure 8 as 6 month mean mortality rates. Both data sets were treated in a product-moment correlation analysis (Table I). Recruitment correlations separate significantly into two groups: station S1 and the shallow site at station G6 as one group and station G4 and the deep site at station G6 as another group (Table I). Thus the archipelago stations S1 and G6, 15 m in the central part of the fjord constitute one reproductively correlated pattern, while G6, 20 m, and station G4 in the inner part of the fjord, constitute another reproductively correlated pattern. However, significant correlation was also

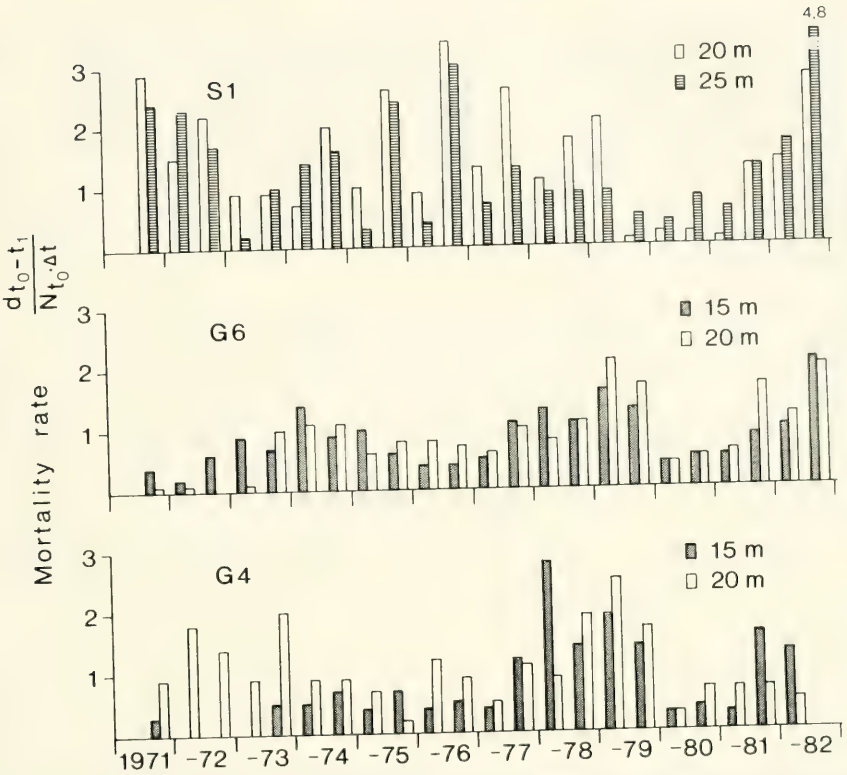


FIGURE 8. Mortality rates of six populations of *Ascidia mentula* at three stations each at two depth levels calculated as six-month means.

found between stations G6 and G4 at both depths, but at a lower level of correlation. In the fjord G6, 20 m, and the shallow site at G4 correlated highly while G4, 20 m, correlated with the other fjord sites less significantly.

Mortality correlations also separated significantly into two groups (Table I). Station S1 is separated and correlated negatively with the fjord stations G4 and G6, indicating that mortality factors were operating differently in the two areas. Stations G6, 20 m, and G4, 15 m, showed significant mortality correlations just as they did with respect to recruitment correlations. Both in recruitment as well as in mortality correlations G4, 20 m, showed some degree of separation from the other fjord stations but G4, 15 m, did not.

Temporal patterns and hydrography

The hydrographic observations (Fig. 2) showed noticeable variations during the 12-year study period. Winter temperatures at both depth levels during 1972–1976 were generally higher than the mean temperatures. In contrast winter temperatures were significantly lower during the years 1977–1982. Similar trends were not observed in the salinity data.

Temporal patterns of recruitment at station S1 and G6, 15 m showed some correlation to hydrography, while station G6, 20 m and G4 had comparatively low recruitment throughout the study period (Figs. 2, 6). Correlation between hydrography and mortality patterns were not apparent.

TABLE I

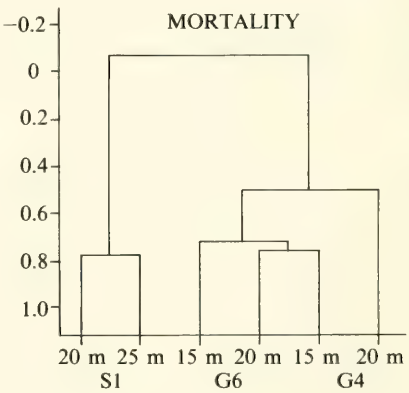
Correlation coefficients and cluster dendrograms of recruitment and mortality rates for A. mentula *at three stations each at two depth levels*

		S1		G6		G4	
		20 m	25 m	15 m	20 m	15 m	20 m
S1	20 m						
	25 m	0.911*					
G6	15 m	0.659*	0.617*				
	20 m	0.388*	0.412*	0.584*			
G4	15 m	0.253	0.251	0.433*	0.878*		
	20 m	0.152	0.177	0.502*	0.797*	0.714*	

* $P < 0.01$

		S1		G6		G4	
		20 m	25 m	15 m	20 m	15 m	20 m
S1	20 m						
	25 m	0.772*					
G6	15 m	−0.079	−0.302				
	20 m	−0.050	−0.147	0.690*			
G4	15 m	0.049	−0.103	0.693*	0.699*		
	20 m	0.083	−0.061	0.347	0.348	0.213	

* $P < 0.01$



DISCUSSION

Recruitment patterns

The positive correlation between recruitment and density may have several explanations:

Attraction of larvae to adults. Attraction of marine invertebrate larvae to adults of the same species occurs in barnacles (Knight-Jones and Crisp. 1953), polychaetes (Wisely, 1960), molluscs (Knight-Jones, 1951), echinoderms (Young and Chia, 1982), and ascidians (Young and Braithwaite, 1980). Larvae of *A. mentula* may occasionally settle on the cuticle of adults but the great majority settle on the substratum among adults. Preliminary experiments failed to demonstrate any attractive effect of adult ascidians on larvae when separated by a plankton-net. If adults attract larvae chemotactically the stimulus must be weak. Figure 4B shows that the recruitment rate increased with population density up to about 100 ind/m^2, above which the rate decreased. The possibility that at high adult densities larvae may be trapped, and consequently destroyed, in the branchial sac of the adults cannot be ruled out.

Local settlement and dispersal. Thorson (1964) suggested that the larval life of ascidians is so short that they will remain mainly in the surroundings where they

were born and so settle among their parents. The correlation analysis (Table I) suggests that recruitment took place locally.

The length of the planktonic larval life of *A. mentula*,and many other solitary ascidians evidentally lasts no more than a few hours and probably only a few minutes. Thorson (1946), did not find significant ascidian larvae among the ½ million planktonic larvae identified in his comprehensive study of the larvae of bottom invertebrates of the Øresund. In the group called "other larvae of bottom invertebrates" which constituted 4.5% (see Thorson, 1946, p. 373), ascidian larvae did not seem to be of importance. Ascidians are, however, common in the Øresund (Dybern, 1967; Lützen, 1967).

Along the Swedish west coast and in the fjords where ascidians are quantitatively abundant Erikson (1973) and Ölundh (1977) rarely found ascidian larvae. The same condition obtained in the Gullmarsfjord plankton (Lönning, 1962; Lindahl and Hernroth, 1983; Hernroth, pers. comm.) although adult populations of several thousand per m^2 can be found (Svane, 1983). Dybern (1965) did find larvae of *Ciona intestinalis* (L.) in plankton samples from a shallow (3–5 m), narrow sound. He concluded that these larvae were from local populations of considerable size. Lützen (1960), in an attempt to collect larvae of the winter breeding *Styela rustica* (L.) in the Gullmarsfjord during its peak reproductive season, found only 27 larvae among several liters of plankton examined.

The length of the pelagic life of ascidian larvae varies, ranging from a few hours to several days (Millar, 1971). In cultures at 15°C in constant dim light where the only substrate was plankton-net or PVC-aquarium walls, the planktonic larval life of *A. mentula* ranged from a few minutes to 240 hours. Fifty percent of the larvae had metamorphosed after 120 hours. When offered a suitable granite rock larval life was considerable reduced. The larvae seemed potentially able to metamorphose shortly after hatching. The swimming ability of *A. mentula* larvae was, however, very poor and no uniform directional movements towards a light source could be recognized (pers. obs. To be reported in detail elsewhere). In nature, therefore, the distribution of larvae must be governed by water movements. Swimming ability may play a role only very close to the substratum. Other species (*Ciona intestinalis, Corella parallelogramma, and Pyura tessellata*) have a shorter larval life under similar laboratory conditions.

The dispersal of recruits occurs first by passive transport of eggs and, after hatching, by passive transport and active swimming of larvae. When an egg is released into sea water the follicle cells expand osmotically and increase their buoyancy (Berrill, 1975). The eggs, however, must be kept in suspension by water movements to avoid sinking. The development time of the eggs is temperature-dependent (Goodbody, 1974, Berrill, 1975) *e.g.*, 30 hours at 15°C (pers. obs.). Therefore, distribution of the larvae of solitary oviparous ascidians, must occur mainly during the period from egg spawning to hatching.

Protection of postlarvae and juveniles. Large populations may protect postlarvae and juveniles. Buss (1981) showed that the bryozoan *Bugula turrita* settled gregariously and preferentially in selected locations where resident density was high or intermediate and that the interspecific competitive ability of *B. turrita* was density-dependent. Dense aggregates of *A. mentula* to some extent prevent sea urchin activity by providing a barrier and thereby reducing mortality within aggregates. Continuous recruitment into established aggregates may prolong longevity in that single individuals may be more easily removed from the substratum. The recruitment rate, however, was density-dependent (Fig. 4B) but the mortality rate density-independent (Fig. 4C). Consequently, this result does not support the idea of Buss (1981) that formation of aggregates in

sessile organisms is caused by density-dependence in settlement reflected by density-dependence in mortality.

At station S1 unoccupied substratum always seemed available, apparently due to sea urchin activity. At station G6 open or unoccupied substratum was sparse but at the 15 m level high densities and high recruitment was observed (Figs. 4, 7). Likewise at station G4 unoccupied substratum was apparently sparse but the epifaunal cover of other organisms was somewhat less than at G6. Some sea urchin activity at the fjord stations was observed but it was considerably less than that at station S1. Figure 4B may indicate that at station G6, 15 m, and S1, 25 m, where recruitment rates were relatively low compared to the high population densities, substratum was a limiting factor during most of the period of study.

Light and substratum orientation. Light infuenced settlement of marine invertebrate larvae (Thorson, 1964) and some ascidians may respond in their settling behavior to light (Grave, 1935; Crisp and Ghobashy, 1971), but many do not (Grave, 1941; Thorson, 1964; Young and Braithwaite, 1980). Ascidians are generally found in shaded situations *e.g.,* the underside of rocks and boulders and below overhangs on rock walls (Dybern, 1963; Crisp and Ghobashy, 1971). At station S1, *A. mentula* is most commonly found below overhangs, somewhat less frequently on vertical rock-surfaces, and not at all on horizontally orientated substrates. Preferential settlement on shaded substrates may explain this pattern, but sea urchin activity may have a similar effect. At the fjord stations G6 and G4, where sea urchin activity was less, *A. mentula* was more evenly distributed. Yet even here *A. mentula* is not found on horizontal substrates. Sedimentation on horizontal substrates may restrict recruitment and subsequent survivorship.

Finally, orientation of the substratum to light and water currents may be important in settlement as in barnacles (Crisp and Barnes, 1954; Crisp and Stubbings, 1957). Station G6, where the highest population density was found at the 15 m level, is situated in the central and most narrow part of the Gullmarsfjord (Fig. 1). The vertical rock walls are densely covered with filter-feeding organisms and large fluctuating populations of the annual *Ciona intestinalis.*

Synchrony in recruitment

Synchrony in settling of intertidal barnacles and molluscs can occur over distances of hundreds of kilometers (*e.g.,* Lewis *et al.,* 1982), although considerable local variations both in space and time can occur (Lewis and Bowman, 1975; Southward, 1967). Keough (1983) found no synchrony in recruitment of sessile invertebrates onto pannels at two subtidal sites in southern Australia and regarded recruitment as a stochastic process. Recruitment of *A. mentula* showed variations among stations and depths (Fig. 7) but correlation analysis revealed a distinct pattern grouping the archipelago sites with the mid-fjord shallow site and the inner fjord sites with the mid-fjord deep site (Table I). The hydrographical properties of the fjord may play an important role in the separation. Mixing of coastal, "North Sea water" with fjord water at the depths in question may occur less frequently in the inner parts of the fjord, especially when the common westerly winds blow surface water into the fjord, where it become relatively isolated.

Reproduction

The reproductive pattern of *A. mentula* reported by Svane and Lundälv (1981) is consistent with the results from this study, at depths greater than 15 m where reproduction occurs throughout the year (Figs. 5, 6). However, at the 15 m levels,

reproduction was seasonal (Fig. 5) with no significant recruitment during winter months. This suggests that larvae may settle at the same depth level as the spawning populations from which they originally arose (Fig. 6).

Temperature is important in gonad maturation and larval development in ascidians (Goodbody, 1974; Berrill, 1975). Mean winter temperatures were considerably lower at 15 m than at 20 m and summer temperatures were somewhat higher at 15 m compared to 20 m (Fig. 2). Comparatively lower winter temperatures and high summer temperatures seemed to induce the seasonal reproductive pattern. Maximum recruitment intensity within the year was similar at all stations and depths, with the exception of G4, 20 m, where maximum intensity took place in July-August. However, it was comparatively low throughout the period of study (Fig. 6).

Mortality

No relationship was found between the mean population density and mortality rate (Fig. 4C) and consequently mortality was density-independent. Possibly density-independent factors operated during the transition from warm to cold periods, with density-dependent factors operating during other periods (Figs. 2, 8). The temperature change in 1977 may have influenced the mortality at the exposed station S1 but no effect was observed at the sheltered stations G6 and G4. Equally, the increased mortality observed at the sheltered stations during 1978–1979, where temperatures were below the mean (Fig. 2), was not observed at station S1 although mortality was relatively high at the 20 m level. When separating the individual stations and depths no correlation was found between yearly mean mortality rate and population density.

The gradient of exposure from station S1 towards the fjord stations was reflected in the mortality rates although within the fjord the rates were higher at the inner station G4 than at station G6 in the mid-fjord.

No substantial predation on *A. mentula* was observed, either in the photographs or during the numerous dives (Svane, 1983). A detailed study of survivorship based on single generations was not meaningful at most stations due to continuous recruitment. At station S1, 25 m, however, the generation time was relatively short and separation of generations was possible. Svane and Lundälv (1981) studied this population during an 8-year period and found that increased mortality correlated with increased reproductive activity but dislocation and disturbance by sea urchins may sometimes be an important mortality factor.

Mortality factors operated differently at different stations. At station S1, where sea urchin activity and a harsh physical environment play important roles, mortality was high. At station G4 and especially at station G6 mortality was comparatively low. The local environment may play an important role. Station G6 is located in the most narrow part of the fjord (Fig. 1) and prevailing currents may provide better food supply. In the fjord in general large sea urchins are sparse and their activity is limited due to dense epifaunal cover.

Temporal patterns and hydrography

Power spectra, from Fourier analysis, of mean annual sea temperatures from the Plymouth area demonstrated a dominating 5-year cycle (Maddock and Swann, 1977). This cycle may be related to the 5-year cycle in monthly mean temperatures from Bornö Hydrographical Station shown in Figure 2. The temperature pattern and the shift in monthly mean temperatures during 1976–1977 was consistent with temperature recordings from the deepest part (700 m) of the Norwegian Trench (Norwegian Fisheries

Board, Flödvigen) and was therefore representative for most of the North Sea and Northeast Atlantic.

The impact of the "warm period" during 1972–1976 on benthic communities in the North Sea area has been shown by Buchanan *et al.* (1978) and Beukema *et al.* (1978) but is not apparent in the long-term investigations of the German Bight presented by Zigelmeier (1978). Gray and Christie (1983) reviewed some long-term studies of benthic populations showing an increase in density during the "warm period" but attributed these changes to 6–7 year cycles. They concluded that many species do respond to long-term hydrographic cycles.

Species with southerly distribution patterns should benefit from elevated winter temperatures and increase in numbers and northerly distribution (Southward *et al.*, 1975). *A. mentula* has a boreal-lusitanian distribution and should thus be favored. The population densities in Figure 3 may be attributed to this pattern. All populations increased in numbers during the "warm period," with some minor fluctuations, and then gradually decreased during the following "cold period."

Recruitment was intensive during the "warm period" (1972–1975) at stations S1 and G6, 15 m, with very high numbers in 1975 (Fig. 7). At stations G6, 20 m, and G4 no important recruitment, however, was recorded during the first four years of the "warm period" but recruitment increased during 1975, 1976, and 1977. During the preceeding "cold period" important recruitment was observed at all stations in a somewhat scattered pattern and was most intensive at stations G6, 15 m, and S1, 25 m. Evidently, recruitment could not be entirely related to temperature variations. Some trends, however, should be noted. The high recruitment in 1975 coincided with unusually high mean temperatures throughout the year. Lower temperatures observed in January 1976 and spring 1979 and the first "cold period" winter temperatures in 1977 (Fig. 2) may be traced in the recruitment patterns (Fig. 7) because very little recruitment took place during these periods.

However, mortality showed no apparent correlation to hydrography and the fjord stations varied in a somewhat contradictory pattern when compared with the archipelago station S1 (Fig. 8). During the cold winter and spring 1978–1979, however, elevated mortality rates were observed at stations S1, 20 m, G6, and G4 but during 1980–1981 mortality was low at all stations and depths with no apparent correlation to temperature or salinity (Figs. 2, 8).

Large-scale temperature cycles and local variations may influence the population density of *A. mentula* by increased recruitment at elevated temperatures but sheltered populations may respond differently. Mortality of adults was regulated locally and temperature independent within the range discussed.

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LITERATURE CITED

BERRILL, N. J. 1950. *The Tunicata.* The Ray Society, London. 354 pp.

BERRILL, N. J. 1975. Chordata: Tunicata. Pp. 241–282 in *Reproduction of Marine Invertebrates* II, Giese, A. C. and J. S. Pearse, eds. Academic Press, New York.

BUSS, L. W. 1981. Group living, competition, and the evolution of cooperation in a sessile invertebrate. *Science* **213:** 1012–1014.

BUCHANAN, J. B., M. SHEADER, AND P. F. KINGSTON. 1978. Sources of variability in the benthic macrofauna off the south Northumberland coast, 1971–1976. *J. Mar. Biol. Assoc. U.K.* **58:** 191–209.

BEUKEMA, J. J., W. DE BRUIN, AND J. J. M. JANSEN. 1978. Biomass and species richness of the macrobenthic animals living on the tidal flats of the Dutch Wadden Sea: long-term changes during a period with mild winters. *Neth. J. Sea Res.* **12:** 58–77.

CHALMER, P. N. 1982. Settlement patterns of species in a marine fouling community and some mechanisms of succession. *J. Exp. Mar. Biol. Ecol.* **58:** 73–85.

CRISP, D. J., AND H. BARNES. 1954. The orientation and distribution of barnacles at settlement, with particular reference to surface contour. *J. Anim. Ecol.* **23:** 142–162.

CRISP, D. J., AND A. F. A. A. GHOBASHY. 1971. Responses of the larvae of *Diplosoma listerianum* to light and gravity. Pp. 443–465 in *4th European Marine Biology Symposium.* Crisp, D. J., ed. Cambridge University Press, London.

CRISP, D. J., AND H. G. STUBBINGS. 1957. The orientation of barnacles to water currents. *J. Anim. Ecol.* **26:** 179–196.

DAYTON, P. K. 1971. Competition, disturbance, and community organization: the provision and subsequent utilization of space in a rocky intertidal community. *Ecol. Monogr.* **41:** 351–389.

DEAN, T. A. 1981. Structural aspects of sessile invertebrates as organizing forces in an estuarine fouling community. *J. Exp. Mar. Biol. Ecol.* **53:** 163–180.

DYBERN, B. I. 1963. Biotope choice in *Ciona intestinalis* (L.). Influence of light. *Zool. Bidrg. Uppsala* **35:** 589–601.

DYBERN, B. I. 1965. The life cycle of *Ciona intestinalis* (L.) *f. typica* in relation to the environmental temperature. *Oikos* **16:** 109–131.

DYBERN, B. I. 1967. The distribution and salinity tolerance of *Ciona intestinalis* (L.) *f. typica* with special reference to the waters around southern Scandinavia. *Ophelia* **4:** 207–226.

ERIKSSON, S. 1973. Abundance and composition of zooplankton on the west coast of Sweden. *Zoon* **1:** 113–123.

FIELD, B. 1982. Structural analysis of fouling community development in the Damariscotta river estuary, Maine. *J. Exp. Mar. Biol. Ecol.* **57:** 25–33.

GIESE, A. C., AND J. S. PEARSE. 1974. Methods of estimating sexual reproductive activity. Pp. 9–14 in *Reproduction of Marine Invertebrates* I, A. C. and J. S. Pearse, eds. Academic Press, New York.

GOODBODY, I. 1974. The physiology of ascidians. *Adv. Mar. Biol.* **12:** 1–129.

GRAVE, C. 1935. Metamorphosis of ascidian larvae. *Pap. Tortugas Lab.* **29:** 209–292.

GRAVE, C 1941. The eyespot and light responses of the larvae of *Cynthia partita. Biol. Bull.* **81:** 287.

GRAY, J. S., AND H. CHRISTIE. 1983. Predicting long-term changes in marine benthic communities. *Mar. Ecol. Prog. Ser.* **13:** 87–94.

KARLSON, R. 1978. Predation and space utilization patterns in a marine epifaunal community. *J. Exp. Mar. Biol. Ecol.* **31:** 225–239.

KAY, A. M., AND M. J. KEOUGH. 1981. Occupation of patches in the epifaunal communities on pier pilings and the bivalve *Pinna bicolor* at Edithburgh, South Australia. *Oecologia* **48:** 123–130.

KEOUGH, M. J. 1983. Patterns of recruitment of sessile invertebrates in two subtidal habitats. *J. Exp. Mar. Biol. Ecol.* **66:** 213–245.

KNIGHT-JONES, E. W. 1951. Gregariousness and some other aspects of setting behaviour of *Spirorbis. J. Mar. Biol. Assoc. U.K.* **30:** 201–222.

KNIGHT-JONES, E. W., AND D. J. CRISP. 1953. Gregariousness in barnacles in relation to the fouling of ships and to anti-fouling research. *Nature* **117:** 1109.

LEWIS, J. R., AND R. S. BOWMAN. 1975. Local habitat-induced variations in the population dynamics of *Patella vulgata* L. *J. Exp. Mar. Biol. Ecol.* **17:** 165–203.

LEWIS, J. R., R. S. BOWMAN, M. A. KENDALL, AND P. WILLIAMSON. 1982. Some geographical components in population dynamics: possibilities and realities in some littoral species. *Neth. J. Sea Res.* **16:** 18–28.

LINDAHL, O., AND L. HERNROTH. 1983. Phyto-zooplankton community in coastal waters of Western Sweden—an ecosystem off balance? *Mar. Ecol. Prog. Ser.* **10:** 119–126.

LUNDÄLV, T. 1971. Quantitative studies on rocky-bottom biocoenoses by underwater photogrammetry. A methodological study. *Thalassia Jugoslav.* **7:** 201–208.

LÜTZEN, J. 1960. The reproductive cycle and larval anatomy of the ascidian *Styela rustica* (L.). *Vidensk. Meddr. Dansk Naturh.* **123:** 227–236.

LÜTZEN, J. 1967. Saekdyr. *Danm. Fauna,* Vol. 75, Gads Forlag, Copenhagen. 267 pp.

LÖNNING, S. 1962. Some observations on plankton from the Gullmar fjord. *Arc. Zool.* **16**(2): 9–40.

MADDOCK, L., AND C. L. SWANN. 1977. A statistical analysis of some trends in sea temperature and climate in the Plymouth area in the last 70 years. *J. Mar. Biol. Assoc. U.K.* **57:** 317–338.

MENGE, B. A. 1976. Organization of the New England rocky intertidal community: role of predation, competition, and environmental heterogeneity. *Ecol. Monogr.* **45:** 355–393.

MILLAR, R. H. 1966. Tunicata: Ascidiacea. In *Marine Invertebrates of Scandinavia,* No. 1, Universitetsforlaget, Oslo.

MILLAR, R. H. 1971. The biology of ascidians. *Adv. Mar. Biol.* **9:** 1–100.

MOUNTFORD, M. D. 1962. An index of similarity and its application to classificatory problems. Pp. 43–50 in *Progress in soil zoology,* P. W. Morphy, ed. Buttersworth, London.

OSMAN, R. W. 1977. The establishment and development of a marine epifaunal community. *Ecol. Monogr.* **47:** 37–63.

SCHOENER, A., AND C. H. GREENE. 1981. Comparison between destructive and nondestructive sampling of sessile epibenthic organisms. *Limnol. Oceanogr.* **26:** 770–774.

SOKAL, R. R., AND F. J. ROHLF. 1981. *Biometry* 2nd ed. W. H. Freeman, San Francisco. 571 pp.

SOUTHWARD, A. J. 1967. Recent changes in abundance of intertidal barnacles in south-west England: a possible effect of climatic deterioration. *J. Mar. Biol. Assoc. U.K.* **47:** 81–95.

SOUTHWARD, A. J., E. I. BUTLER, AND L. PENNYCUICK. 1975. Recent cyclic changes in climate and in abundance of marine life. *Nature* **253:** 714–717.

SUTHERLAND, J. P. 1981. The fouling community at Beaufort, North Carolina: A study in stability. *Am. Nat.* **118:** 499–519.

SUTHERLAND, J. P., AND R. H. KARLSON. 1977. Development and stability of the fouling community at Beaufort, North Carolina. *Ecol. Monogr.* **47:** 425–446.

SVANE, I. 1983. Ascidian reproductive patterns related to long-term population dynamics. *Sarsia* **68:** 249–255.

SVANE, I., AND T. LUNDÄLV. 1981. Reproductive pattern and population dynamics of *Ascidia mentula* O. F. Müller on the Swedish west coast. *J. Exp. Mar. Biol. Ecol.* **50:** 163–182.

SVANE, I., AND T. LUNDÄLV. 1982a. Population dynamics and reproductive patterns of *Boltenia echinata* (Ascidiacea) on the Swedish west coast. *J. Neth. Sea Res.* **16:** 105–118.

SVANE, I., AND T. LUNDÄLV. 1982b. Persistence stability in ascidian populations: Long-term population dynamics and reproductive pattern of *Pyura tessellata* (Forbes) in Gullmarsfjorden on the Swedish west coast. *Sarsia* **67:** 249–257.

SVANSSON, A. 1975. Physical and chemical oceanography of the Skagerrak and the Kattegat. *Rept. Fish. Bd. Sweden, Inst. Mar. Res.,* No. 1. 88 pp.

THORSON, G. 1946. Reproduction and larval development of Danish marine bottom invertebrates. *Medd. Komm. Havundersoeg. Kbh., Ser. Plankton* **4:** 1–523.

THORSON, G. 1964. Light as an ecological factor in the dispersal and settlement of larvae of marine bottom invertebrates. *Ophelia* **1:** 167–208.

TORLEGÅRD, K., AND T. LUNDÄLV. 1974. Under-water analytical system. *Photogramm. Eng.* **40:** 287–293.

WISELY, B. 1960. Observations on the settling behaviour of larvae of the tubeworm *Spirorbis borealis* Daudin (Polychaeta). *Aust. J. Mar. Freshw. Res.* **11:** 55–72.

YOUNG, C. M., AND L. F. BRAITHWAITE. 1980. Larval behavior and post-settling morphology in the ascidian, *Chelyosoma productum* Stimpson. *J. Exp. Mar. Biol. Ecol.* **42:** 157–169.

YOUNG, C. M., AND F.-S. CHIA. 1982. Factors controlling spatial distribution of the sea cucumber *Psolus chitonoides:* Settling and post-settling behaviour. *Mar. Biol.* **69:** 195–205.

ZIEGELMEIER, E. 1978. Macrobenthos investigation in the eastern part of the German Bight from 1950 to 1974. *Rapp. P.-v. Réun. Int. Explor. Mer* **172:** 432–444.

ÖLUNDH, E. 1977. A comparative study of three zooplankton communities on the Swedish west coast with respect to composition, dynamics, and function. Ph.D. Thesis, Götebg. Univ.

Reference: *Biol. Bull.* **167:** 647–657. (December, 1984)

ENVIRONMENTAL CONTROL OF AMYLASE PHENOTYPE IN AMPHIPODS OF THE GENUS *GAMMARUS*

RICHARD BOROWSKY

Department of Biology, New York University, Washington Square, New York 10003

ABSTRACT

The amylases of three species of *Gammarus* amphipods, *G. palustris, G. mucronatus,* and *G. lawrencianus,* were studied using polyacrylamide gel electrophoresis. In all three species there are two principal zones of activity (*Amy-1* and *Amy-2*), but occasional individuals exhibit a third band (*Amy-X*). *Amy-X* variants are more common in the deeper water species and in collections made during late winter or early spring than in the shallower water species or in summer collections. Experimental work shows that *Amy-X* can be induced by a diet rich in amylose or glycogen, but not by amylopectin or other foods, and by reducing the temperature at which they are maintained. It is hypothesized that the relative expressions of *Amy-X* and *Amy-1* vary seasonally, and that this variation is an adaptive response to seasonal variation in diet, or conditions for the hydrolysis of substrate.

INTRODUCTION

One of the least understood phenomena in population biology is how the environment influences the genetic contents and phenotypic expressions of natural populations. Amphipods of the genus *Gammarus* are useful in the study of this question because they are distributed among a variety of aquatic environments, ranging from freshwater, through estuarine, to marine. *Gammarus* species found in the intertidal zone in marine and estuarine environments can be easily collected in large numbers and tend also to be hardy enough to culture in the laboratory. Thus, data on gene frequencies in natural populations can be obtained easily, and hypotheses relating to the effects of environmental factors on the population can be tested by laboratory experiment. Furthermore, the amphipods are an intrinsically interesting group for evolutionary studies because they are believed to be currently in the midst of an explosive adaptive radiation (Bousfield, 1973).

This paper reports work on amylase variation in populations of *Gammarus palustris, G. mucronatus,* and *G. lawrencianus.* These three species are estuarine to marine in distribution, co-occur at a number of localities in the New York area, and are zoned ecologically in areas of sympatry, from the high intertidal through the low intertidal-subtidal, in the order listed above. Amylase was chosen for study because the enzyme acts upon a heterogeneous substrate set which is externally derived. Environmental variation is expected to influence isozyme variation at such loci more than at loci coding for enzymes which act upon internally derived homogeneous substrate sets (Gillespie and Kojima, 1968; Kojima, *et al.,* 1970; Johnson, 1973, 1975)

Amylases in these species are coded by at least two distinct loci (*Amy-1* and *Amy-2*) and can easily be resolved by electrophoresis on polyacrylamide gels. In occasional individuals of all three species, a third band (*Amy-X*), running close to *Amy-1,* is

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clearly exhibited. This band was initially ignored, because of its unreliable appearance, until it was observed that collections made during colder weather yielded a higher proportion of *Amy-X* individuals than those made during warmer weather. This observation suggested that differential expression of amylase isozymes in *Gammarus* could be controlled by environmental variation. Therefore, a series of laboratory experiments was performed in order to identify environmental factors that might influence the expression of *Amy-X*. Temperature and diet were the two factors chosen.

Materials and Methods

All animals used in these experiments were collected at low tide in the intertidal zone at two sites in Brooklyn, New York. *Gammarus palustris* and *G. mucronatus* were taken at the Cross Bay Boulevard Bridge, (mean salinity, 2.4%) at the head of Jamaica Bay and *G. lawrencianus* was taken at Knapp Street, (mean salinity, 3.0%). Collections were made during 1977–1979. Experimental animals were maintained in the laboratory in filtered water from the collection sites on a 12:12 light dark cycle for times and at temperatures specified in Table I. Holding (acclimation) temperatures before the experiments were chosen to match collection temperatures.

The foods used in the feeding experiments were: *Ulva lactuca* or *Enteromorpha intestinalis* ("green algae"), *Punctaria sp* (= "brown algae"), commercially available shrimp meat, and four artificial foods: (1) agarose flakes (Indubiose A45, L'Industrie Biologique Francaise), (2) amylopectin (Sigma #A-8515)-agarose flakes (2 parts amylopectin to 1 part agarose), (3) amylose (Sigma #A-0512)-agarose flakes (2:1), and (4) mussel glycogen (Sigma #G1508)-agarose flakes (2:1). The flake foods were prepared by dissolving the constituents in boiling water, coating a glass plate with the solution, drying the gel, and scraping off the film.

Properties of the artifical foods

All four of the flake foods maintained their overall structure in water for the durations of the feeding experiments. Separate experiments were performed in order to determine whether the starches would leach out of the films into solution. Fifty milligram portions of the different films were added to 10 ml portions of distilled water and the mixtures were sampled after 1, 24, and 48 hours. The agarose film and its soak gave negative results when tested with Gram's iodine reagent. The amylose soak was iodine negative after 1 and 24 hours but gave a weak positive reaction after 48 hours. The amylopectin soak gave a strong reaction after only 1 hour, but the film retained some amylopectin and gave a moderate reaction even after 48 hours in water. The glycogen chosen gives only a weak reaction with iodine so it was not tested. Glycogens, however, are of low solubility in cold water. The amphipods fed avidly upon the three flake foods containing starch or glycogen. They also were observed to feed upon the agarose film, but much less of it was eaten.

Two clarifications of usage should be made here. First, the term "induction" is used in its broadest sense in this paper, to describe the phenomenon in which environmental factors lead to the production of a new enzyme form. Its usage is not meant to imply any particular molecular mechanism out of the several possibilities which are considered in the discussion. Second, the induction of *Amy-X* was followed experimentally in terms of the expression of *Amy-X relative* to that of *Amy-1*. That is, increases in the relative expression of *Amy-X* were ascribed solely to its induction, and the effect of "repression" of *Amy-1* was ignored. This convention, choosing *Amy-1* as the fixed reference, was adopted because absolute activities cannot easily be read

from amylase zymograms, and because variation in "staining intensity" among individuals is far greater for *Amy-X* than for *Amy-1*. In particular, *Amy-1* activity is almost invariably present, while *Amy-X* activity is often absent, and the probability of its presence is clearly influenced by the treatments described below. Experiment 11 addressed this assumption directly and showed that changes in the intensity of both isozymes do occur, although the changes are greater for *Amy-X*.

Electrophoretic and staining techniques

Freshly sacrificed amphipods were homogenized in 20 to 150 microliters of buffer (0.01 *M* tris-Cl, pH 7.6, 0.001 *M* beta-mercaptoethanol and 0.001 *M* EDTA) and the homogenates were cleared by centrifugation (10,000 × *g*, 10 min). Supernatant was used directly for electrophoresis or for enzyme assays. Samples (10 microliters) were mixed with an equal volume of 40% sucrose in water containing bromphenol blue as a tracking dye, and electrophoresed (PAGE) in polyacrylamide slab gels (7.2%T:2.5%C) using the Ornstein-Davis buffer system (Smith, 1968) for 1.5 to 2.5 hours at 20 to 25 mA. Relative mobility values (RM) were calculated with respect to the migration of tracking dye.

Amylase activity was visualized by incubating the gel on a "starch plate" subsequently developed with a Gram's iodide/iodine solution (2 g KI, 1 g I_2, in 300 ml. H_2O). The "starch plates" were prepared by coating a "gelbond" sheet (Marine Colloids, Inc.) to a thickness of 1 mm with starch solution (1% soluble starch or amylopectin, 1% agarose in 0.02% NaCl, 0.02 *M* tris-HCl pH 7.0) and evaporating it to dryness.

The relative expression of *Amy-1* and *Amy-X* isozymes was scored visually on a scale of 1 to 7. Samples exhibiting activity in only one of the two zones were rated either "1" (*Amy-1*) or "7" (*Amy-X*). Equal expression of both bands was scored "4," just noticeable differences, "3" or "5," while great inequalities, "2" or "6."

This method of scoring the gels was chosen because of the practical difficulties involved in obtaining quantitative activity data from the developed starch plates. The starch coatings were not uniform in thickness among plates, and even on the same plate the intensity of the background iodine staining was variable. Furthermore, the amount of activity for each isozyme varied among animals, so to visualize all of the samples run on a given gel, the starch plates were over-incubated. This procedure brought many of the samples, which surely differed in activity, to the identical end point, achromaticity. For these reasons, the relative activities of bands were taken in most of the experiments as ordinal, rather than rational, variables. In experiment 11, for reasons detailed below, densitometry was employed, although the quantitative data obtained were used only for descriptive purposes.

Statistics

Because the data consisted of ordinal variables, non-parametric statistics were used for all tests: contingency was tested using Chi Squared statistics, or Fisher's exact test when one or more cells had low expected values. The effects of diet and temperature were tested for significance using Wilcoxon Statistics, or the Fisher test, when applicable.

RESULTS

Zones of amylase activity resolved by PAGE

Two principal zones of amylase activity were resolved in all three species by PAGE. The more anodal zone (*Amy-1*) has an RM of about 0.9, while the slower

TABLE I

The effects of diet and temperature on the relative expression of Amy-1 *and* Amy-X*

Replicate number	Time and temperature	Feeding: Control 1	Control 2	Brown	Unfed	Agarose	Amylose Agarose	Amylopectin Agarose	Shrimp	Amylose statistics
1	2D, 22°C 1D, 22°C		2.1 ± 0.4 8		1.4 ± 0.5 8		4.1 ± 2.0 7	3.3 ± 2.0 9		T = 37.5 *P* = .036
2	20D, 22°C 5D, 22°C	1 ± 0 8	1.5 ± 0.6 4	1.5 ± 0.6 4	1 ± 0 5	1 ± 0 5	3.0 ± 0.7 5			T = 16 *P* = .002
3	6D, 6°C 2D, 6°C		1.2 ± 0.5 5		1.1 ± 0.4 7	1.4 ± 0.5 7	3.0 ± 1.3 6	1.2 ± 0.5 5		T = 18.5 *P* = .041
4A	1D, 5°C 3D, 5°C		4.6 ± 1.3 5	4.1 ± 0.8 8	4.2 ± 0.8 5	4.9 ± 0.8 8	4.4 ± 0.9 5	4.4 ± 1.1 5		*P* = NS
4B	Same 4D, 5°C		3.6 ± 0.6 5	3.8 ± 0.5 5	3.4 ± 0.6 5	4.6 ± 1.1 5	5.6 ± 0.6 5	3.8 ± 1.3 5		T = 15 *P* = .008
5A	21D, 14°C 1–2D, 4°C	1 ± 0 5	2.2 ± 0.4 6				3.0 ± 1.3 7			*P* = NS
5B	Same 1–2D, 22°C		1 ± 0 6				2.7 ± 1.6 7			T = 10 *P* = .029
6A	>60D, 22°C 1D, 4°C	2.2 ± 1.1 5	2.0 ± 0 5				2.2 ± 0.5 5			*P* = NS

6B	Same 2D, 4°C		4.3 ± 1.3 4			5.8 ± 0.5 5			P = NS
7A	5D, 10°C 1–6D, 10°C		1.9 ± 0.5 4	1.9 ± 0.6 8				2.1 ± 0.4 7	
7B	Same **		1.2 ± 0.5 4					3.5 ± 1.0 4	
8A	3D, 10°C 7D, 10°C			1.9 ± 2.2 5	1.6 ± 0.7 9	5.3 ± 1.0 10	1.2 ± 0.4 9		
8B	Same ***		1.9 ± 0.3 9			3.9 ± 1.6 4	2.0 ± 0.7 4	2.0 ± 0.2 10	T = 23 P = .006
9	**** 1–2D, 11°C	1.3 ± 0.5 6				2.3 ± 1.0 6	1.1 ± 0.4 8		T = 28 P = .094
10	>60D, 20°C 3D, 10°C						4.3 ± 2.1 7	5.8 ± 1.5 9	

* Mean indices of expression ± one standard deviation are listed on the first line for each replicate, sample sizes on the second line. The first lines in the second column list the holding temperatures and times (in days) prior to the experimental treatments, while the second lines give the durations and temperatures of the experimental treatments. "Control 1" animals were sacrificed at the start of the experiment, "Control 2" animals were maintained on green algae for the duration, and "Unfed" animals were starved for the duration. The other diets are described in the text. All animals were maintained on green algae prior to the experiment, with supplements of shrimp meat in replicates 6, 7, and 9. Wilcoxon T statistics for the comparisons of Amylose fed and Control 2 animals and their associated two-tailed probabilities are listed in the last column.

** Starved 4D, then refed for 1–2D, 10°C.

*** Starved 6D, then refed 1D, 10°C.

**** 4D, then starved 8D, 11°C. T test compares "Amylose" and "Control 1."

zone (*Amy-2*) has an RM of about 0.55. In all cases, whole organism extracts exhibited far more activity in zone 1 than in zone 2. In each zone, extracts of individual animals exhibited either one or two bands. Progeny analyses of sibships are consistent with the hypothesis that double banding of *Amy-2* results from heterozygosity and single banding from homozygosity (R. Borowsky, unpub.). This is generally true for amylase in other animal species (Karn and Malacinski, 1978), true in the isopod *Asellus aquaticus* (Lomholt and Christensen, 1970), and is assumed here to be so for *Amy-1* also. Banding patterns, double *versus* single, were uncorrelated between zones, and the zones, therefore, appear to be coded for by different loci.

The third band (*Amy-X*, RM = .87) is the focus of this paper, and was exhibited weakly by some individuals. The expression of *Amy-X* in field caught animals was generally so poor that there was little likelihood of mistaking an individual with *Amy-X* activity for an *Amy-1* heterozygote.

Amy-X in natural populations

The frequency of field collected *Amy-X* variant individuals differed among species and season of collection. For individuals collected from July through September, the frequencies of *Amy-X* variants were 0 out of 216 in *G. palustris*, 2 out of 105 in *G. mucronatus*, and 19 out of 66 in *G. lawrencianus* (Heterogeneity $\chi^2 = 16.34$, df = 2, $P < .01$, ignoring contributions from the smallest cells; overall $\chi^2 = 85.2$). That is, *Amy-X* was most common in the deeper water species and least common in the species with the highest distribution. For individuals collected during March and April, shortly after these species returned from overwintering in deeper waters, the frequency of *Amy-X* was 24 out of 41 in *G. palustris* and was 4 out of 12 in *G. lawrencianus*. The difference in frequency of *Amy-X* between seasons of collection is highly significant for the *G. palustris* sample ($P < .001$, Fisher's exact test), although not for the *G. lawrencianus* sample. All of these animals had *Amy-1* activity.

The apparent seasonal variation in the occurrence of *Amy-X* in *G. palustris* implies that the *relative amounts* of activity in the *Amy-X* and *Amy-1* zones are controlled by environmental factors and suggests the hypothesis that *Amy-X* is a "deep water" or "cold water" isozyme, while *Amy-1* is a "shallow water" or "warm water" isozyme.

Induction of Amy-X by diet and temperature

In order to determine whether the expression of *Amy-X* could be influenced by environmental conditions, ten experiments were performed in which diet and maintenance temperature were varied. *Gammarus palustris* (n = 347) were used for these experiments, and the relative expression of *Amy-1* and *Amy-X* was scored by visual inspection of the stained starch plates after PAGE. Table I specifies the experimental conditions and lists the results as mean scores ± standard deviations.

The results can be summarized as follows: A diet of amylose-agarose increased expression of *Amy-X* over control values in ten of eleven experimental subsets (expts. 1–6, 8, and 9: sign test, two-tailed $P = .012$). In six instances, the individual differences were significant or highly significant (Table I).

Agarose alone in the diet had no effect on the expression of *Amy-X* (4 cases, expts. 2–4 and 8). Neither did starvation for the brief periods employed (one to seven days, expts. 1–4, 7, and 8), nor brown algae in the diet (expts. 2 and 4). In experiment 7b, shrimp meat significantly increased the expression of *Amy-X* (Fischer's exact two-tailed $P = .029$), but in experiment 8 it had no effect. Although shrimp in the diet is associated with prominent expression of *Amy-X* in experiment 10, this experiment

was inadequately controlled and the effect of shrimp must be considered unresolved. Amylopectin-agarose failed to increase the relative expression of *Amy-X* in five experiments (1, 3, 4, 8, and 9).

Reduction in temperature also appears to induce *Amy-X*. In experiment 5, animals were maintained on green algae for three weeks at 14°C and then shifted either to 4° or 22°C. Half were retained on a diet of green algae and the other half were shifted to amylose-agarose. A control group was sacrificed prior to the changes in conditions. In the set retained on green algae, increase in temperature had no effect on *Amy-X* expression while decrease in temperature increased its expression (4° *vs.* 22°C control 2 groups; Wilcoxon $T_{6,6} = 21$, $P = .0022$). The set shifted to amylose-agarose exhibited increased expression of *Amy-X* regardless of temperature, although those shifted to low temperature exhibited better expression than those shifted to high temperature. In experiment 6, juveniles that had been born and raised in the laboratory on a diet of green algae and shrimp meat at 22°C were switched to 4°C and either green algae or amylose-agarose. A control group was sacrificed at the time the experimentals were shifted and the experimentals were sacrificed after one and two days. No differences in *Amy-X* expression were found in the experimental group one day after the shift, but in both feeding groups *Amy-X* expression was significantly increased by the second day (Amylose 6A *vs.* 6B, $T_{5,5} = 15$, $P = .008$; control 1 *vs.* 6B control 2, $T_{4,5} = 12$, $P = .064$: 6A *vs.* 6B control 2 groups; $T_{4,5} = 10$, $P = .016$). While the increased expression can be attributed to diet in the amylose-agarose group, it must be attributed to the temperature change in the control group.

An eleventh feeding experiment was performed in order to determine the effect of mussel glycogen in the diet. *Gammarus palustris* were collected in the field and transferred to filtered water at 19°C for four days. During that period one group was fed agarose flakes, another was fed amylose-agarose, and the third was fed glycogen-agarose. At the end of the feeding period the animals were frozen whole and lyophilized. Six pooled samples of five animals each were made up for each of the three treatments and the samples were weighed (dry) and ground in buffer at a ratio of 1:20 (W:V). The samples were cleared by centrifugation, and 20 microliters of each were used for electrophoresis. Because of the uniform dilution and the equal sample sizes, semi-quantitive comparisons among bands on the stained plate were possible in this experiment.

The agarose fed animals expressed *Amy-X* poorly, if at all. In contrast, both the amylose and the glycogen fed animals clearly expressed *Amy-X*. Therefore, glycogen feeding also induces the synthesis of *Amy-X*. Of greatest interest, however, is the observation that the overall intensity of *Amy-1* banding in the amylose treatment was uniformly lighter than that in the other treatments (Fig. 1). This shows that amylose treatment effects a true differential induction of *Amy-X* over *Amy-1* instead of simply increasing levels of both isozymes to a point at which the *Amy-X* band can be discerned on the plate.

Is Amy-X *an artifact?*

Takeuchi *et al.* (1975) have shown that the electrophoretic mobility of rat liver amylase depends upon whether the source animal had been well-fed or fasted prior to sacrifice. Amylase from fasted animals had a greater anodal mobility than amylase from well fed animals. This difference proved to be due to glycogen bound to the amylase of well fed rats and the effect was duplicated *in vitro* by mixing amylase from fasted rats with 4% glycogen, prior to electrophoresis. Such a phenomenon

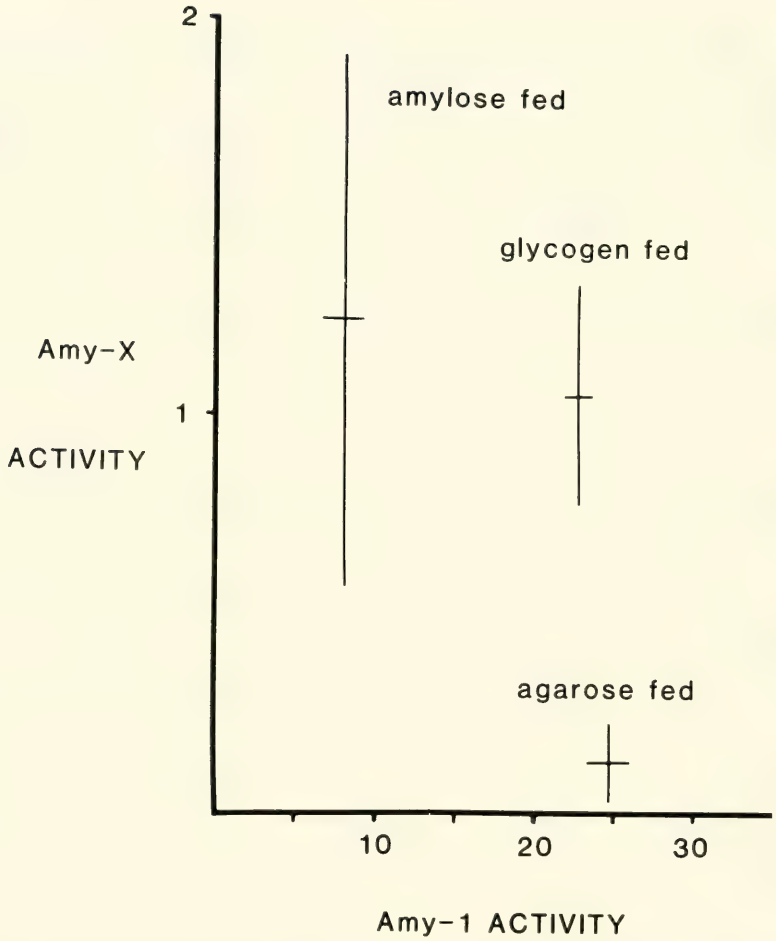

FIGURE 1. Relative activities of *Amy-1* and *Amy-X* depend upon diet in *G. palustris*. Activity is expressed in arbitrary units proportional to the area under each peak obtained by densitometry (Gelman instruments) of the original starch plate used for experiment 11. Each point is the mean of the six samples run (five for amylose) and the bars denote one S.E. of the mean.

might account for the generation of *Amy-X* from *Amy-1* in starch and glycogen fed *Gammarus*, especially since the mobility of *Amy-X* is lower than that of *Amy-1*.

In order to test whether substrate binding could account for the "induction" of *Amy-X*, an experiment was performed in which equal volumes of the same samples were electrophoresed in the presence and the absence of glycogen. Ten microliters of each of five samples from experiment 11 were mixed with equal volumes of water prior to electrophoresis while another set of the same samples were run after mixture with equal volumes of 8% mussel glycogen in water. Glycogen had no effect on electrophoretic mobilities or the relative distribution of activity between *Amy-1* and *Amy-X* in the samples, although the staining intensities of the samples pre-mixed with glycogen were uniformly less intense than those of the samples diluted with water.

DISCUSSION

The data establish three things: first, there are two amylase isozymes of high anodal mobility in *Gammarus,* and, in *Gammarus palustris,* they can be induced differentially. At high temperatures (14°–22°C) and on a diet of green macro-algae and shrimp, *Amy-1* is the predominant fast running isozyme and *Amy-X* is present only in trace quantities; lowering the temperature to 4°C or feeding a diet high in amylose or glycogen causes the differential induction of *Amy-X.* Second, field caught *G. palustris* individuals express *Amy-X* activity with higher frequency in the winter and spring than in the summer and fall. This suggests a seasonal pattern of variation in amylase phenotype. Third, in summer and fall collections of the three species, the expression of *Amy-X* was greater in *G. lawrencianus* than in *G. mucronatus,* and least in *G. palustris.* This suggests an ecological zonal pattern of variation.

The experimental results are consistent with the observations on field caught animals and suggest ways to account for observed variation among seasons and species. The apparent seasonality in expression of amylase observed in *G. palustris,* for example, could be accounted for by seasonal variation in temperature. Low temperatures during the winter would enhance the expression of *Amy-X* phenotype. It is possible, also, that a seasonal variation in diet could be a contributing factor. Gut content analyses show that *G. palustris* feeds year-round (Gable and Croker, 1977), and also that their diet varies seasonally. While the major portion of their diet year-round consists of unidentifiable detritus, during the winter the identifiable fraction contains a greater proportion of non-filamentous green algae and lower proportions of diatoms and blue-green algae than during the spring. Thus, either qualitative or quantitative differences among seasons in the amounts of starches and glycogens available could modify the effects of temperature. If intertidal zone diets differed in general from subtidal diets this might also account for the differences observed among species. Because of their zonation, *G. lawrencianus* is more exposed to deeper water food sources than is *G. mucronatus* which, in turn, has a greater exposure than *G. palustris.*

Differential amylase induction in *Gammarus* might function to adapt the organism to seasonal variation in temperature and the structure of available substrates. Differential isozyme induction has been cited before as a potential mechanism for temperature acclimatization (Hochachka and Somero, 1968; Somero, 1969a), and the concept can be broadened to cover adaptation to variation in any type of environmental factor, including substrate structure. If the enzyme form induced by a given set of environmental conditions has catalytic properties optimizing function under those conditions, the induction would be adaptive, both physiologically and evolutionarily.

A clear example is provided by acetylcholinesterase isozymes in rainbow trout brain tissue (Baldwin and Hochachka, 1970; Baldwin, 1971). One acetylcholinesterase isozyme is induced after acclimatization at high temperatures (17°C), a different form is induced at low temperatures (2°C), and both forms are present at intermediate temperatures. The differential induction appears to be an adaptive response optimizing the efficiency of catalysis over a temperature range, because the isozymes have temperature dependent K_m curves with minima at their induction temperatures. An analogous situation has been reported for pyruvate kinase in the Alaskan King-Crab by Somero (1969a), although in this case the two enzyme forms are apparently coded for by a single locus and are interconvertible. Other examples of temperature-dependent differential enzyme induction in fish and invertebrates are provided by Flowerdew and Crisp (1976), Kent and Hart (1976), and Marcus (1977).

Amy-X is not a simple Mendelian allelic variant of *Amy-1* and its presence is clearly inducible. While it is possible that *Amy-X* and *Amy-1* are coded for by different loci, this is by no means certain. Evidence is accumulating that protein diversity can result from variable processing of RNA, or post-translational modification of polypeptides. Furthermore, such modifications have been documented for amylase systems. Kikkawa (1964), for example, has shown that each major amylase band from *Drosophila melanogaster* is associated with a minor band, and both are coded for by the same allele. MacDonald *et al.* (1977) have demonstrated that amylase mRNA from dog pancreatic tissue is translated *in vitro* into a polypeptide 1500 Daltons larger than the native enzyme. This suggests post-translational modification of the protein. In mice, amylase mRNA from both liver and salivary tissue exhibits a heterogeneity caused by variable processing (Hagenbuchle *et al.,* 1981). Craik *et al.* (1983) have proposed that variable excision of introns from genes such as amylase could account for polypeptide diversity. It is reasonable to suppose that environmental conditions could affect the splice position, and result in the observation of "inducibility." Whether or not *Amy-X* and *Amy-1* are coded for by the same locus, the observations reported here are of importance because they link the phenomenon of differential inducibility to specific environmental factors that are of relevance in nature.

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LITERATURE CITED

BALDWIN, J. 1971. Adaptation of enzymes to temperature: Acetylcholinesterases in the central nervous system of fishes. *Comp. Biochem. Physiol.* **40B:** 181–187.

BALDWIN, J., AND P. W. HOCHACHKA. 1970. Functional significance of isoenzymes in thermal acclimatization. *Biochem. J.* **116:** 883–887.

BOUSFIELD, E. L. 1973. *Shallow Water Gammaridean Amphipoda of New England.* Comstock Publishing, Ithaca.

CRAIK, C. S., W. J. RUTTER, AND R. FLETTERICK. 1983. Splice junctions: Association with variation in protein structure. *Science* **220:** 1125–1129.

FLOWERDEW, M. M., AND D. J. CRISP. 1976. Allelic esterase isozymes, their variation with season, position on the shore and stage of development in the cirripede *Balanus balanoides. Mar. Biol.* **35:** 319–325.

GABLE, M. F., AND R. A. CROKER. 1977. The salt marsh amphipod, *Gammarus palustris* Bousfield, 1969 at the northern limit of its distribution. *Estuarine Coastal Mar. Sci.* **5:** 123–134.

GILLESPIE, J. H., AND K. KOJIMA. 1968. The degree of polymorphisms in enzymes involved in energy production compared to that in nonspecific enzymes in two *Drosophila ananassae* populations. *Proc. Natl. Acad. Sci. U.S.A.* **61:** 582–585.

HAGENBUCHLE, O., M. TOSI, U. SCHIBLER, R. BOVEY, P. K. WELLAUER, AND R. A. YOUNG. 1981. Mouse liver and salivary gland alpha-amylase mRNAs differ only in 5′ non-translated sequences. *Nature* **289:** 643–646.

HOCHACHKA, P. W., AND G. N. SOMERO. 1968. The adaptation of enzymes to temperature. *Comp. Biochem. Physiol.* **27B:** 659–668.

JOHNSON, G. 1973. Importance of substrate variability to enzyme polymorphism. *Nature New Biol.* **243:** 151–153.

JOHNSON, G. 1975. Enzyme polymorphism and adaption. *Stadler Symp.* **7:** 91–116.

KARN, R. C., AND G. M. MALACINSKI. 1978. The comparative biochemistry, physiology, and genetics of animal alpha-amylases. *Adv. Comp. Physiol. Biochem.* **7:** 1–101.

KENT, J. D., AND R. G. HART. 1976. The effect of temperature and photoperiod on isozyme induction in selected tissues of the creek chub *Semotilus atromaculatus*. *Comp. Biochem. Physiol.* **54B**: 77–80.

KIKKAWA, H. 1964. An electrophoretic study on amylase in *Drosophila Melanogaster*. *Jpn. J. Genet.* **39**: 401–411.

KOJIMA, K. J., K. J. GILLESPIE, AND Y. N. TOBARI. 1970. A profile *Drosophila* species' enzymes assayed by electrophoresis. I. Number of alleles, heterozygosities, and linkage disequilibrium in glucose-metabolizing systems and some other enzymes. *Biochem. Genet.* **4**: 627–637.

MACDONALD, R. J., A. E. PRZYBYLA, AND W. J. RUTTER. 1977. Isolation and *in vitro* translation of messenger RNA coding for pancreatic secretory proteins. *J. Biol. Chem.* **252**: 5522–5528.

MARCUS, N. H. 1977. Temperature induced isozyme variants in individuals of the sea urchin, *Arbacia punctulata*. *Comp. Biochem. Physiol.* **58b**: 109–113.

SMITH, I. 1968. Disc electrophoresis on acrylamide gels; Techniques of disc electrophoresis. Pp. 365–389 in *Chromatographic and Electrophoretic Techniques*, Vol. 2, 2nd ed. I. Smith, ed. Interscience Publishers, New York.

SOMERO, G. N. 1969a. Enzymic mechanisms of temperature compensation: immediate and evolutionary effects of temperature on enzymes of aquatic poikilotherms. *Am. Nat.* **103**: 517–530.

SOMERO, G. N. 1969b. Pyruvate kinase variants of the Alaskan King-Crab: evidence for a temperature-dependent interconversion between two forms having distinct and adaptive kinetic properties. *Biochem. J.* **114**: 237–241.

TAKEUCHI, T., T. MATSUSHIMA, AND T. SUGIMURA. 1975. Electrophoretic and immunological properties of liver alpha-amylase of well-fed and fasted rats. *Biochim. Biophys. Acta* **403**: 122–130.

Reference: *Biol. Bull.* **167**: 658–668. (December, 1984)

CHROMOSOME POLYMORPHISM IN *GOBIUS PAGANELLUS*, LINNEO 1758 (PISCES, GOBIIDAE)

R. VITTURI, P. CARBONE*, E. CATALANO, AND M. MACALUSO

*Institute of Zoology, University of Palermo, Italy, and *Institute of Genetics, University of Palermo, Italy*

Abstract

In the present investigation the diploid numbers 2n = 45, 2n = 46, 2n = 47, and 2n = 48 have been determined for *Gobius paganellus*. Specimens of different sex were found to have exactly the same karyotype. This species is characterized by two fundamental numbers: NF = 47, and NF = 48. Chromosome polymorphism due to different chromosome rearrangements within the A-type complement is present in this species.

Introduction

The family Gobiidae is quite interesting because of its controversial morphological features (Fage, 1925; Arai and Sawada, 1974; Nishikawa *et al.*, 1974) and its evolutionary stage which is not completely known (Chen and Ebeling, 1971; Chiarelli and Capanna, 1973; Arai *et al.*, 1974; Manna and Prasad, 1974; Arai and Sawada, 1975; Khuda-Bukhsh, 1978; Colombera and Rasotto, 1982). A high variability of chromosome number occurs within this group ranging from 2n = 40 to 2n = 62 (Sola *et al.*, 1979).

There still is no agreement as to the number of chromosomes characterizing *Gobius paganellus*. The diploid number 2n = 45 was proposed for a female specimen caught in the Thyrrhenian Sea (Cataudella *et al.*, 1973), n = 25 and 2n = 50 for male specimens caught in the Northern Adriatic Sea (Colombera and Rasotto, 1982), and 2n = 46 for eight male and female specimens caught in the Southern Mediterranean Sea near Spanish coasts (Thode *et al.*, 1983).

Thode *et al.*, (1983) claim that *Gobius paganellus* is characterized by male heterogamety (XY). The occurrence of a large metacentric chromosome in the female specimen investigated by Cataudella *et al.* (1973) does not agree with the mechanism of sex determination proposed for this species.

The present investigation was aimed at clarifying these problems by analyzing chromosome sets from numerous male and female *Gobius paganellus*, Linneo 1758, specimens.

Materials and Methods

Twenty-six *Gobius paganellus* specimens (17 females, 7 males, and 2 sexually immature specimens) caught in the Gulf of Palermo were analyzed and classified according to the guidelines of Tortonese (1975) and Bini (1969). Specimens from this study were deposited at the Institute of Zoology of the University of Palermo.

Each specimen was injected intraperitoneally with colchicine (0.1%, 1 ml/30 g body weight) and sacrificed two hours later. Kidney and spleen tissues were removed, and minced in 0.075 *M* KCl. The suspension was centrifuged for twenty minutes

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and the supernatant was discarded, while cell sediment was fixed in two successive changes of fresh methanol-acetic acid (3:1). Drops of cell suspension were then placed on clean slides at 0°C. Slides were prepared by air-drying and stained with 2.5% Giemsa (Gurr, R 66) for 20–30 minutes.

Observations and photographs were made using a Wild or Leitz microscope. Chromosomes were classified according to arm ratio, as proposed by Levan *et al.* (1964). Metacentric (M) chromosomes are referred to as bi-armed chromosomes, while sub-telocentric (ST) and acrocentric (A) chromosomes are referred to as mono-armed chromosomes.

Results

Observations were made on six female specimens and one sexually immature specimen with the modal chromosome number 2n = 48 (Table I). An average karyotype, arbitrarily designated as A-type complement (Fig. 3; Table II), was obtained by measuring and arranging pairs of homologous chromosomes from nine metaphase plates by decreasing length and according to centromere position. These chromosomes cannot be grouped into classes according to length, since their size ranges from 2.67 to 0.84 μm and they are all acrocentrics (Fig. 1a, b) except for one element of the first pair (Fig. 2a, b) which was observed in five specimens, in which AR = 3.13. This chromosome can therefore be classified as sub-telocentric.

Table I

Diploid chromosome counts in 26 specimens of Gobius paganellus

Specimen number	Sex	Number cells analyzed	Diploid counts										
			≤40	41	42	43	44	45	46	47	48	49	≥50
1	♀	18							2	1	15		
2	♀	28	3			1		1			22	1	
3	♀	33	2			1			2		28		
4	♀	30					1	2			25		2
5	♀	30		2					2	1	25		
6	♀	27					2			2	23		
7	?	18					1			2	15		
8	♀	37	3					3	1	30			
9	♂	16	1							15			
10	?	32	3	2						27			
11	♂	27					1		2	23	1		
12	♂	12	1					1		10			
13	♀	13		2				1		10			
14	♀	23				2			1	20			
15	♂	36						2	2	32			
16	♀	28	2					1	3	22			
17	♀	26	4					2		20			
18	♂	30				2		3		25			
19	♂	17		2			1		15				
20	♀	39	2				1	1	35				
21	♀	20	1				1		18				
22	♀	23		1				2	20				
23	♀	20						1	18	1			
24	♀	18	2				1		15				
25	♂	30	2	1			2	25					
26	♀	48	3			3	2	40					
		679											

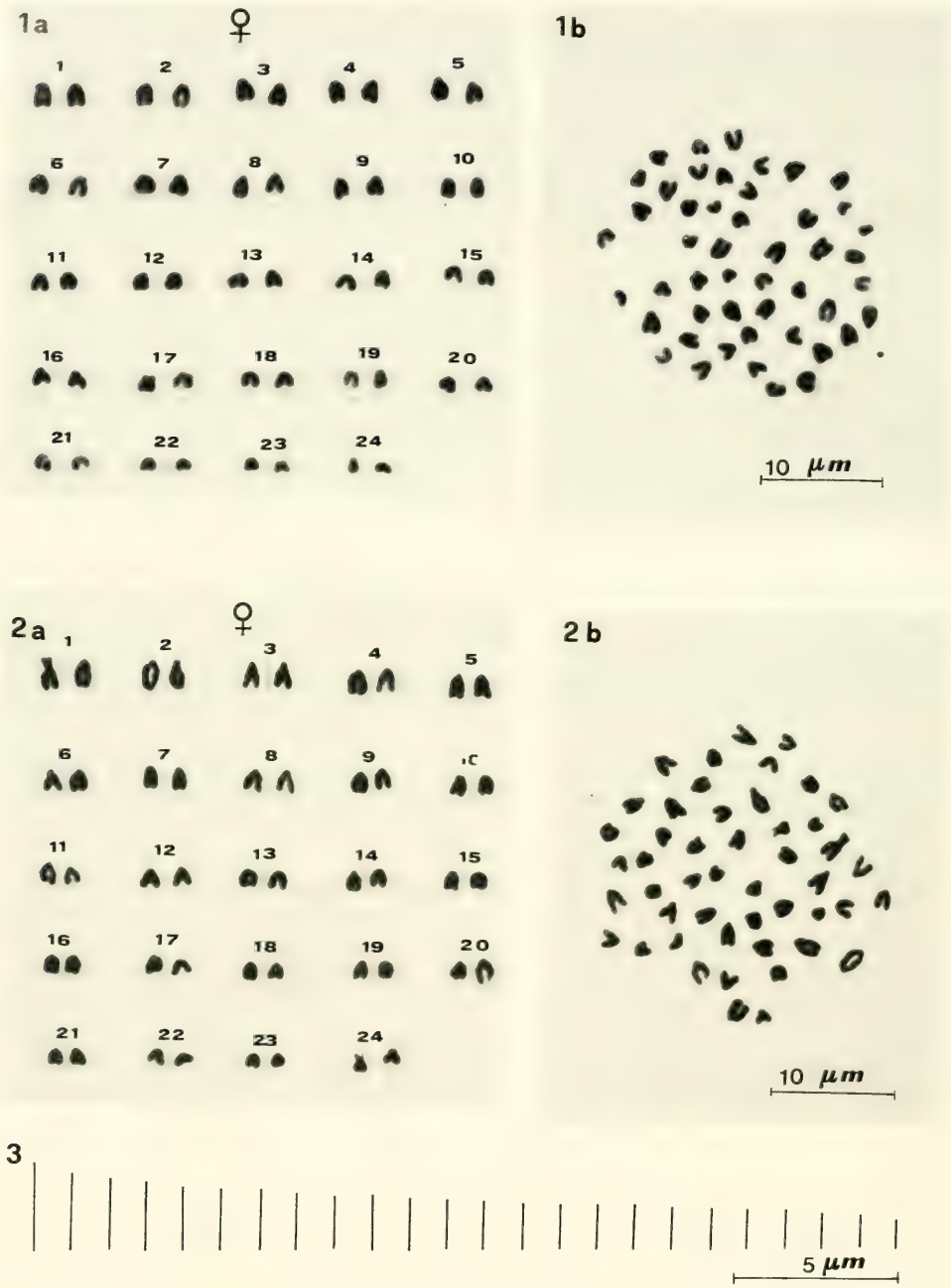


FIGURE 1a, b. Karyotype and metaphase plate of female *Gobius paganellus* (2n = 48).

FIGURE 2a, b. Karyotype and metaphase plate of female *Gobius paganellus* (2n = 48) with one subtelocentric chromosome.

FIGURE 3. Average karyotype obtained from nine metaphase plates of *G. paganellus*.

TABLE II

Mean length and arm ratio of the A-type chromosomes of nine metaphase plates (2n = 48) of Gobius paganellus *(specimens 1–7)*

Chromosome pairs	Mean length in microns ±S.D.	Arm ratio mean	Centromere position
1	2.67 ± 0.24	∝	A
2	2.31 ± 0.32	∝	A
3	2.18 ± 0.26	∝	A
4	2.07 ± 0.20	∝	A
5	2.01 ± 0.22	∝	A
6	1.95 ± 0.26	∝	A
7	1.88 ± 0.26	∝	A
8	1.79 ± 0.28	∝	A
9	1.72 ± 0.26	∝	A
10	1.64 ± 0.20	∝	A
11	1.62 ± 0.22	∝	A
12	1.58 ± 0.22	∝	A
13	1.53 ± 0.24	∝	A
14	1.48 ± 0.22	∝	A
15	1.43 ± 0.24	∝	A
16	1.43 ± 0.24	∝	A
17	1.39 ± 0.24	∝	A
18	1.35 ± 0.20	∝	A
19	1.30 ± 0.22	∝	A
20	1.22 ± 0.17	∝	A
21	1.17 ± 0.17	∝	A
22	1.10 ± 0.20	∝	A
23	0.95 ± 0.14	∝	A
24	0.84 ± 0.05	∝	A

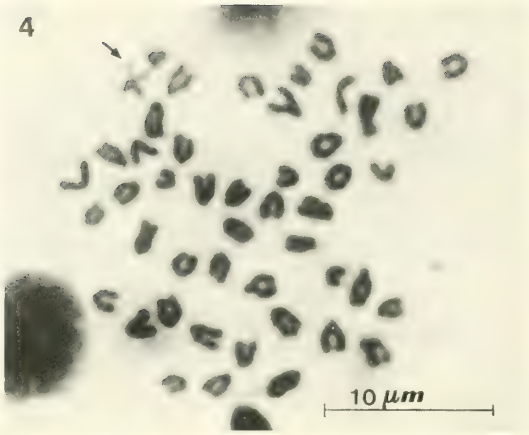
In some specimens with this karyotype a small acrocentric chromosome with satellites was identified with conventional staining (Fig. 4, see arrow). Figure 5 shows the sub-telocentric chromosome (first pair) and the small chromosome with its satellites (24th pair) obtained from different plates.

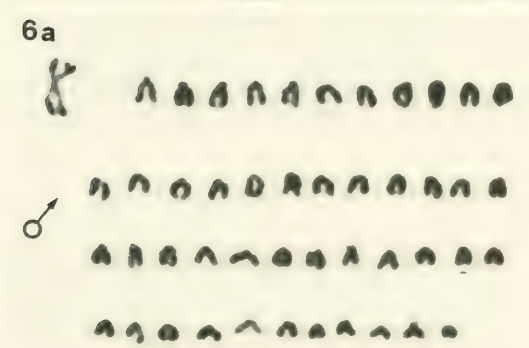
The other nineteen specimens studied differ from those showing an A-type complement not only for the chromosome number (Table I) but also for the presence of one or two bi-armed elements. Some of these specimens show the small satellited acrocentric chromosome.

Average sizes of bi-armed chromosomes were obtained from five different plates for each specimen studied and are reported in Table III. Analysis of average arm ratio in bi-armed chromosomes shows that they are two metacentric chromosomes of different size.

Of the nineteen specimens studied, one male (Fig. 6a, b), one female (Fig. 7a, b), and one sexually immature specimen with 2n = 47 showed MI, but four males (Fig. 8a, b) and four females (Fig. 9a, b) also with 2n = 47 showed element MII. Of the six specimens with 2n = 46, four females (Fig. 10a, b) showed two MII, one female (Fig. 11a, b) only one MII, and one male (Fig. 12a, b) both bi-armed chromosomes MI and MII. Two specimens of different sex (Fig. 13a, b; 14a, b) with 2n = 45 showed MI and MII.

Results summarized in Table IV show that the population of *Gobius paganellus* examined here possessed two fundamental numbers: NF = 47, and NF = 48.





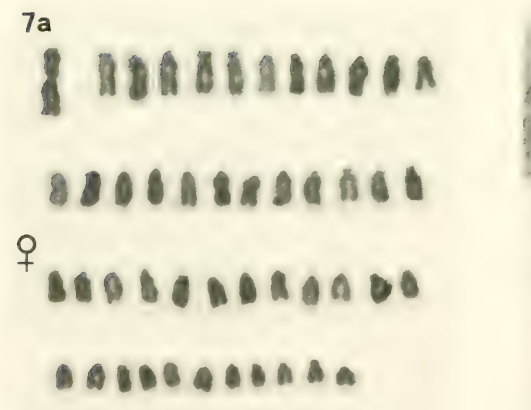


FIGURE 4. Metaphase plate of *G. paganellus*. (Arrow shows the small chromosome with its satellites.)

FIGURE 5. Sub-telocentric chromosome (first pair) and small chromosome (24th pair) obtained from different plates of *G. paganellus*.

FIGURE 6a, b. Alignment of somatic chromosomes and metaphase plate of male *G. paganellus* (2n = 47).

FIGURE 7a, b. Alignment of somatic chromosomes and metaphase plate of female *G. paganellus* (2n = 47).

TABLE III

Average length of bi-armed chromosomes from five metaphase plates for each non-A type specimen of Gobius paganellus *(MI = long bi-armed chromosome; MII = small bi-armed chromosome)*

			No bi-armed chromosome		Mean length in microns ±S.D.		Arm ratio mean	
Specimens	Sex	2n	MI	MII	MI	MII	MI	MII
8	♀	47	1		4.00 ± 0.92		1.34	
9	♂	47	1		3.95 ± 0.70		1.29	
10	?	47	1		5.05 ± 1.25		1.27	
11	♂	47		1		2.18 ± 0.26		1.26
12	♂	47		1		2.25 ± 0.60		1.39
13	♀	47		1		3.27 ± 0.67		1.32
14	♀	47		1		2.86 ± 0.38		1.35
15	♂	47		1		2.84 ± 0.30		1.24
16	♀	47		1		2.73 ± 0.43		1.26
17	♀	47		1		2.50 ± 0.45		1.29
18	♂	47		1		2.39 ± 0.26		1.25
19	♂	46	1	1	4.00 ± 1.17	2.27 ± 0.53	1.26	1.29
20	♀	46		2		2.64 ± 0.26		1.25
21	♀	46		2		2.64 ± 0.65		1.23
22	♀	46		2		2.36 ±0.35		1.34
23	♀	46		2		2.73 ± 0.80		1.31
24	♀	46		1		3.09 ± 0.59		1.27
25	♂	45	1	1	5.50 ± 1	3.06 ± 0.46	1.28	0.37
26	♀	45	1	1	4.59 ± 0.52	2.41 ± 0.26	1.24	1.26

DISCUSSION

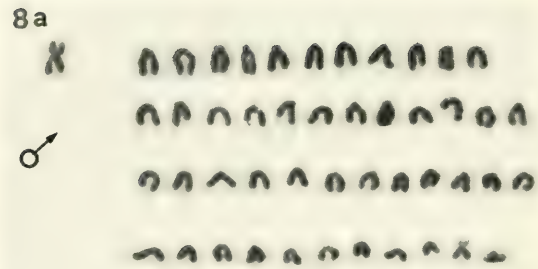
The present investigation was aimed at determining chromosome number in *Gobius paganellus;* modal numbers were found to be 2n = 45, 2n = 46, 2n = 47, and 2n = 48 (Table I). Figures with other than the modal number (aneuploid cells) found in our study were probably artifacts of preparation.

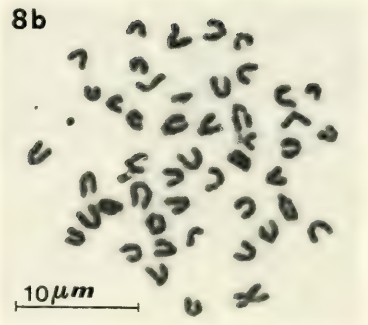
None of the above numbers agrees with 2n = 50, proposed by Colombera and Rasotto (1982) for this species. Values 2n = 45 and 2n = 46, however, confirm those reported by Cataudella *et al.* (1973) and Thode *et al.* (1983), respectively.

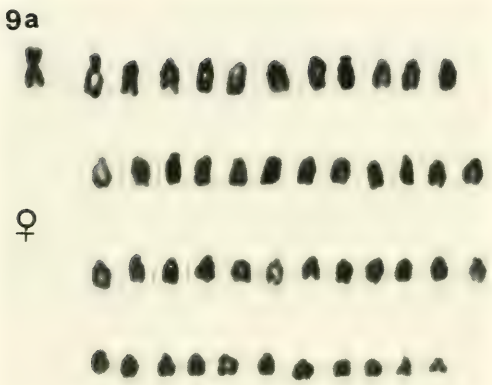
Comparison of chromosome sets observed by the latter authors and those reported in the present paper shows the following substantial differences: (1) Cataudella *et al.* (1973) established that in one female specimen of *Gobius paganellus* the diploid chromosome set 2n = 45 includes only one long metacentric chromosome. In our two specimens with 2n = 45, two bi-armed chromosomes (MI, and MII) (Table III) occurred. (2) Thode *et al.* (1983) reported the diploid value 2n = 46 (NF = 47 in males and NF = 48 in females) in this species and proposed a sex-determining mechanism XX/XY. Such a mechanism is not confirmed by the present research since specimens of different sex were found to have exactly the same karyotype (Table III; Fig. 6a, b; 7a, b; 8a, b; 9a, b; 13a, b; 14a, b)

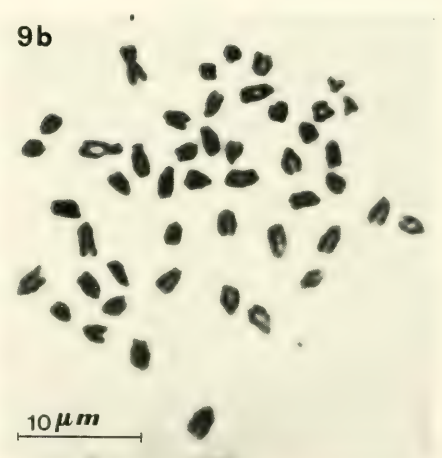
Interestingly enough, only three species (over 50 species studied) of the Gobiidae show heteromorphic sex chromosomes (Nogusa, 1955; Subrahmanyam, 1969; Arai and Sawada, 1974).

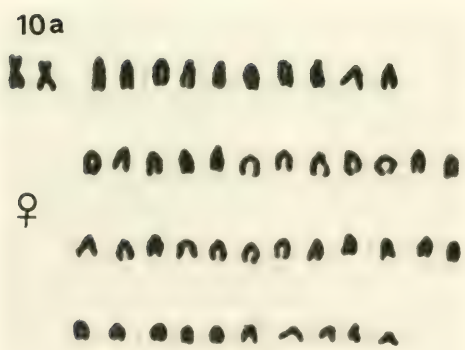
The possibility that chromosome number may vary between different populations of the same species within this family was recently proposed by Colombera and Rasotto (1982). We found such variability in specimens from one popultion of *Gobius paganellus.* We found different diploid chromosome numbers as well as variations











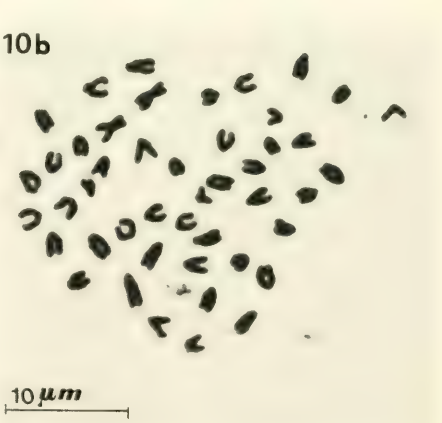
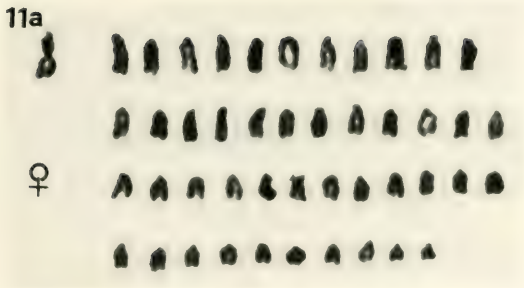
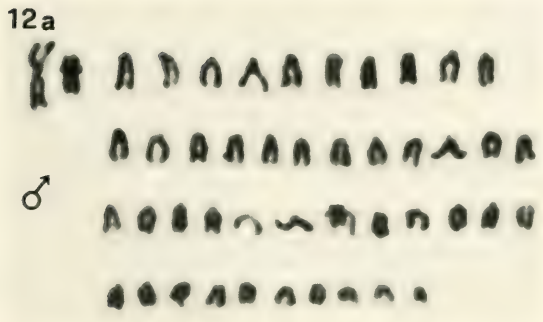


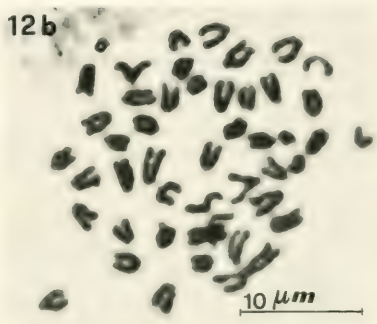
FIGURE 8a, b. Alignment of somatic chromosomes and metaphase plate of male *G. paganellus* (2n = 47).

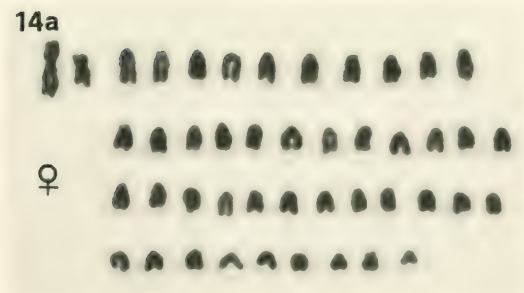
FIGURE 9a, b. Alignment of somatic chromosomes and metaphase plate of female *G. paganellus* (2n = 47).

FIGURE 10a, b. Alignment of somatic chromosomes and metaphase plate of female *G. paganellus* (2n = 46).









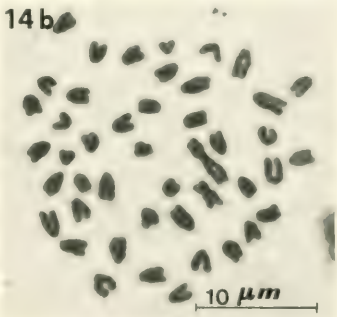


FIGURE 11a, b. Alignment of somatic chromosomes and metaphase plate of female *G. paganellus* (2n = 46).

FIGURE 12a, b. Alignment of somatic chromosomes and metaphase plate of male *G. paganellus* (2n = 46).

FIGURE 13a, b. Alignment of somatic chromosomes and metaphase plate of male *G. paganellus* (2n = 45).

FIGURE 14a, b. Alignment of somatic chromosomes and metaphase plate of female *G. paganellus* (2n = 45).

TABLE IV

Diploid numbers, chromosome morphology, and fundamental number (NF) in Gobius paganellus

Number of specimens	2n	Morphology			NF
		MI	MII	A + ST	
2	45	1	1	43	47
1	46	—	1	45	47
1	46	1	1	44	48
4	46	—	2	44	48
8	47	—	1	46	48
3	47	1	—	46	48
7	48	—	—	48	48
26					

in the chromosome arm number (NF) in this species. With respect to the latter, specimens studied in the present research had either NF = 48 or NF = 47, as shown in Table IV.

According to numerous authors (Ohno *et al.*, 1969; Ebeling and Chen, 1970; Ohno, 1970; Chen, 1971; Thode *et al.*, 1983) who maintain that 48 is the ancestral number in diploid fishes, we propose that the probable "wild type" karyotype of *Gobius paganellus* consists of 2n = 48 acrocentric chromosomes.

In specimens with 2n = 46 and 2n = 47 (NF = 48), the absence of two acrocentric chromosomes with respect to the A-type complement is regularly associated with the presence of one or the other of two metacentric chromosomes. It is clear therefore, that polymorphism for chromosome number is due to the presence of two independent Robertsonian fusions (MI and MII) in the population studied.

There is also a polymorphism for one sub-telocentric element in eight specimens which is probably due to a pericentric inversion.

Fishes with 2n = 46 to 2n = 48, but NF = 48 (Table IV) should be the result of random matings among balanced gametes produced in heterozygotes for each metacentric plus the two acrocentric homologs.

The NF = 47 fish observed here (Table IV), and NF = 50 fish found in other studies (Cataudella *et al.*, 1973) must be aneuploids. Then the variation of chromosome number as well as NF, must be the result of fusion of unbalanced gametes formed in either type of heterozygotes.

Robertson's translocation, pericentric inversion, and loss of chromosome segments are, in fact, considered to be chiefly responsible for chromosomal polymorphism in several species (White, 1969; Chen, 1971; Denton, 1973).

Finally, it should be noted that chromosome polymorphism observed in *Gobius paganellus* is not an isolated finding within the Pisces: it has also been found in the genus *Drascyllus* (Ojima and Kasciwagi, 1981), in the genus *Fundulus* (Chen, 1971; Black and Howell, 1978) and in the genus *Salmo* (Hartley and Horne, 1984 and authors quoted by them).

Examples of polymorphic species are reported in other animal groups (Ford *et al.*, 1957; Bianchi *et al.*, 1969; Lee and Zimmerman, 1969; authors quoted by White, 1969; Bantock and Cockayne, 1975).

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LITERATURE CITED

ARAI, R., AND Y. SAWADA. 1974. Chromosomes of Japanese gobioid fishes I. *Bull. Natl. Sci. Mus. Tokyo* **17**(2): 97–105.

ARAI, R., AND Y. SAWADA. 1975. Chromosomes of Japanese gobioid fishes III. *Bull. Natl. Sci. Mus. Tokyo* **1**(4): 225–239.

ARAI, R., I. KATSUYAMA, AND Y. SAWADA. 1974. Chromosomes of Japanese gobioid fishes II. *Bull. Natl. Sci. Mus. Tokyo* **17**(4): 269–279.

BANTOCK, C. R., AND W. C. COCKAYNE. 1975. Chromosomal polymorphism in *Nucella lapillus*. *Heredity* **34**: 231–245.

BIANCHI, N. O., J. CONTRERAS, AND F. N. DULOUT. 1969. Intraspecies autosomal polymorphism and chromosome replication in *Akodon molinae*. (Rodentia: Cricetidae). *Can. J. Genet. Cytol.* **II**: 233–242.

BINI, G. 1969. *Atlante dei Pesci delle Coste Italiane*. Vol. VII, pp. 109–110.

BLACK, A., AND M. W. HOWELL. 1978. A distinctive chromosomal race of the cyprinodontid fish, *Fundulus notatus*, from the upper Tombigbee River system of Alabama and Mississippi. *Copeia* **2**: 280–288.

CATAUDELLA, S., V. CIVITELLI, AND E. CAPANNA. 1973. The chromosomes of some Mediterranean teleosts: Scarpaenidae, Serranidae, Labridae, Blennidae, Gobiidae (Pisces Scarpaeniformes, Perciformes) *Boll. Zool.* **40**: 385–389.

CHEN, T. R. 1971. A comparative chromosome study of twenty killifish species of the genus *Fundulus* (Teleostei, Cyprinodontidae). *Chromosoma (Berl.)* **32**: 436–455.

CHEN, T. R., AND A. W. EBELING. 1971. Chromosomes of the goby fishes in the genus *Gillichthys*. *Copeia* **1**: 171–174.

CHIARELLI, A. B., AND E. CAPANNA. 1973. *Cytotaxonomy and Vertebrate Evolution*. Academic Press, London-New York.

COLOMBERA, D., AND M. RASOTTO. 1982. Chromosome studies in males of *Gobius niger jozo* (Padoa) and *Gobius paganellus* (Linneo) (Gobiidae, Osteichtyes). *Caryologia* **35**(2): 257–260.

DENTON, T. E. 1973. *Evolution of the Fish Karyotype. Fish Chromosoma Methodology*. Charles C. Thomas Publisher, Illinois. Pp. 129–148.

EBELING, A. W., AND T. R. CHEN. 1970. Heterogamety in teleostean fishes. *Trans. Am. Fish. Soc.* No. 1, **99**: 131–138.

FAGE, L. 1925. Rémarqués sur l'évolution des gobiidae accomagnees d'un synopsis des éspecies europeennes. *Arch. Zool. Exp. Gen.* **55**: 17–34.

FORD, C. E., J. L. HAMERTON, AND G. B. SHARMAN. 1957. Chromosome polymorphism in the common shrew. *Nature* **180**: 392–393.

HARTLEY, S. E., AND M. T. HORNE. 1984. Chromosome polymorphism and constitutive heterochromatin in the Atlantic salmon, *Salmo salar*. *Chromosoma (Berl.)* **89**: 377–380.

KHUDA-BUKHSH, A. R. 1978. Somatic chromosomes of an estuarine fish, *Trypauchen vagina* (Fam. Gobiidae) from Sagar Island, West Bengal, India. *Curr. Sci.* **47**(4): 137–138.

LEE, M. R., AND E. G. ZIMMERMAN. 1969. Robertsonian polymorphism in the cotton rat, *Sigmodon fulviventer*. *J. Mammalol.* **50**: 333–339.

LEVAN, A., K. FREDGA, AND A. A. SANDBERG. 1964. Nomenclature for centromeric position of chromosomes. *Hereditas* **52**: 201.

MANNA, G. K., AND R. PRASAD. 1974. Chromosome analysis in three species of fishes belonging to family Gobiidae. *Cytologia* **39**: 609–618.

NISHIKAWA, S., K. AMAOKA, AND K. NAKANISHI. 1974. A comparative study of chromosomes of twelve species of Gobioid Fishes in Japan. *Jpn. J. Ichthyol.* **21**(2): 61–71.

NOGUSA, S. 1955. Chromosome studies in Pisces IV. The chromosomes of *Mogrunda obscura* (Gobiidae), with evidence of male heterogamety. *Cytologia* **20**: 11–18.

OHNO, S. 1970. The enormous diversity in genome sizes of fish as a reflection of nature's extensive experiments with gene duplication. *Trans. Am. Fish. Soc.* **99**(1): 120–130.

OHNO, S., J. MURAMOTO, C. STENIUS, L. CHRISTIAN, W. A. KITTRELL, AND N. B. ATKIN. 1969. Microchromosomes in Holocephalian, chondrostean and holostean fishes. *Chromosoma (Berl.)* **26:** 35–40.

OJIMA, Y., AND E. KASHIWAGI. 1981. Chromosomal evolution associated with Robertsonian fusion in the genus *Drascyllus* (Chrominae, Pisces). *Proc. Jpn. Acad.* **57**(Ser. B.) 368–370.

SOLA, L., S. CATAUDELLA, AND E. CAPANNA. 1981. New developments in vertebrate cytotaxonomy. III. Karyology of bony fishes: a review. *Genetica* **54:** 285–328.

SUBRAHMANYAM, K. 1969. A karyotype study of the estuarine fish *Boleophtalmus boddaerti* (Pallas) with calcium treatment. *Curr. Sci.* **38**(18)**:** 437–439.

THODE, G., J. CANO, AND M. C. ALVAREZ. 1983. A karyological study on four species of Mediterranean Gobiid fishes. *Cytologia* **48:** 131–138.

TORTONESE, E. 1975. *Fauna d'Italia. Osteichthyes—pesci ossei.* Calderini Publisher, Italia.

WHITE, M. J. D. 1969. Chromosomal rearrangements and speciation in animals. *Annu. Rev. Genet.* **3:** 75–98.

Reference: *Biol. Bull.* **166**: 669–682. (December, 1984)

NUTRIENT TRANSLOCATION IN THE SEA STAR: WHOLE-BODY AND MICROAUTORADIOGRAPHY AFTER INGESTION OF RADIOLABELED LEUCINE AND PALMITIC ACID

F. B. BEIJNINK AND P. A. VOOGT

Research Group for Invertebrate Reproductive Physiology, Laboratory of Chemical Animal Physiology, State University of Utrecht, 8 Padualaan, 3508 TB Utrecht, The Netherlands

ABSTRACT

Using the sea star, *Asterias rubens*, whole-body autoradiography has been employed to follow the distribution and the pathways of translocation of both soluble and tissue-incorporated label derived from orally administered ^{14}C-labeled leucine or palmitic acid. Radioactivity remains localized predominantly in the stomach and pyloric caeca until sixteen days after ingestion. Labeling of the perivisceral coelomic cavity in regions close to the stomach shortly after ingestion points to initial displacement of ingested nutrients through the coelomic fluid and coelomocytes. After oral administration of labeled palmitic acid, distinct labeling of the gastric hemal tufts, axial organ, and aboral ring prior to labeling of the gonads also suggests the involvement of hemal tissue and surrounding perihemal coelomic sinuses in storage and translocation of substances needed for gamete nutrition.

Microautoradiography of gonad tissue reveals a rapid labeling of the walls of the genital coelomic sinus, the ground substance of the genital hemal sinus, and, after prolonged incubation, the germinal epithelium. Little or no label is incorporated into the outer sac of the gonad wall.

The results are discussed in terms of current knowledge on nutrient translocation in the sea star.

INTRODUCTION

Several physiological studies during the last two decades have investigated the pathways and mechanisms of nutrient translocation in the sea star. Various techniques have been employed to follow transfer of nutrients from digestive and storage organs (pyloric caeca) to peripheral areas of the body. Such techniques are generally based on oral administration of radiolabeled nutrients or non-nutritive tracers. Subsequent sampling from coelomic compartments possibly involved in translocation reveals rapid transport of small amounts of tracer from the digestive system to the perivisceral coelomic cavity (Ferguson, 1964a) and the (perihemal) axial sinus (Broertjes *et al.*, 1980b). The nature and the amount of radioactive substances in several internal organs at various times after ingestion of a radiolabeled precursor can be determined using extraction and analytical procedures (Oudejans and Rutten, 1982; Oudejans *et al.*, 1983). The results of these studies also indicate transfer through the coelomic cavity and a possible involvement of coelomocytes in lipid transport. By oral administration—or infusion into the axial sinus—of a vital dye, Broertjes and Posthuma (1978) and Broertjes *et al.* (1980a) demonstrated the possibility of translocation of substances from stomach to gonads through the hemal system; this system would be

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operative especially in conveying high-molecular substances needed for vitellogenesis (Broertjes *et al.*, 1980b).

Up to now, autoradiography of orally administered radiolabeled nutrients in sea stars has been performed in three studies. Ferguson (1963) studied autoradiographs prepared from sections of the rays of *Asterias forbesi* fed with clams which had been injected with various radioactive organic compounds. He concluded that the perivisceral fluid and other body fluids were primarily responsible for nutrient translocation; no evidence indicating a transport role for the hemal system or coelomocytes was found. Broertjes *et al.* (1980b) demonstrated radioactivity in deeper layers of the digestive epithelium of the pyloric stomach and the associated hemal tissue of *Asterias rubens* after ingestion of radiolabeled leucine. During the preparation of this paper, a systematic study was published in which the distribution of radioactivity in digestive system, body wall, hemal organs, and gonads of *Echinaster* was delineated autoradiographically at various times after ingestion of radiolabeled amino acids (Ferguson, 1984); results were interpreted as indicative of a role of hemal tissue in translocation of nutritive substances to the gonads and parts of the tube feet.

Since the autoradiographic studies mentioned above have all been performed on Bouin-fixed paraffin sections of small pieces of specific organs, they do not provide information on the overall distribution of radiolabel throughout the asteroid body or on the distribution of labeled compounds that are lost during fixation and dehydration procedures (*e.g.*, amino acid not incorporated into proteinoid material). In the present study we employed whole-body autoradiography to follow the fate of ingested ^{14}C-labeled L-leucine and palmitic acid in *Asterias rubens*. This technique combines good resolution with the possibility of interpretation of the overall distribution in whole-body sections and allows localization of soluble compounds as it is applied to unfixed frozen tissue. Microautoradiography on semi-thin sections of gonad tissue was performed simultaneously in order to obtain more detailed information on the pathways of translocation of substances needed for gonad development.

Materials and Methods

Animals

Mature specimens of *Asterias rubens* were collected in the Dutch Wadden Sea. The animals were kept at natural daylength in a flow-through sea water system at 12°C and fed *ad libitum* with the sea mussel *Mytilus edulis*. Specimens with body weights from 70 to 100 g and arm-lengths of approximately 7 cm (measured from the center of the disc) were used. The experiments were performed during active gametogenesis in the period of December 1982 to February 1983.

Radioactive precursors

L-[^{14}C(U)]-leucine (13.1 GBq/mmol) and [^{14}C(U)]-palmitic acid (29.6 GBq/mmol) were obtained from New England Nuclear (Dreieich, W. Germany). L-leucine was dissolved in 3% NaCl and palmitic acid was dissolved in 3% NaCl containing 5% bovine serum albumin. Each animal received a final volume of 0.2 ml of precursor solution (total radioactivity 0.37 MBq = 10 μCi).

Experimental procedure

Sea stars were temporarily immobilized in a 1:1 mixture of 3% $MgCl_2$—blocking neuromuscular transmission—and sea water for 10 minutes. This procedure prevented the adambulacral spines from covering the peristome and thus facilitated access to the esophagus. Precursor was administered with a microsyringe fitted with a poly-

ethylene tube to prevent injury of the stomach. Sea stars were laid upside down and, after careful insertion of the tube into the esophagus, the precursor solution was injected very slowly into the stomach. Five minutes after removal of the syringe, the animals were put in fresh sea water. After 25 minutes, each sea star was placed separately in a large beaker, containing 1500 ml of fresh sea water which was aerated continuously. The temperature of the sea water was maintained at approximately 13°C. The animals were allowed to feed on specimens of *Mytilus edulis*.

Autoradiography

At 1.5, 6, and 24 hours, and at 4 and 16 days (in pilot experiments also at 4 hours and 6 days) after ingestion of the precursor, animals were immobilized in 3% $MgCl_2$ for 5 min. Animals were then frozen by submersion in hexane cooled with solid carbon dioxide (−75°C), wrapped in polyethylene bags, and stored in sealed boxes at −60°C up to 4 months. Other specimens were dissected after immobilization; small fragments of the gonads were fixed in 3% glutaraldehyde in 0.2 *M* sodium cacodylate buffer (pH 7), containing NaCl (30 mg/ml) and $CaCl_2$ (2 μg/ml), for 2 h at 4°C (Walker, 1979). Postfixation in 1% OsO_4 for 2 h at 4°C was followed by dehydration in ethanol and propylene oxide and embedding in Epon 812/Araldite 506.

For whole-body autoradiography, frozen animals were embedded in carboxymethylcellulose gel and sectioned in a LKB 2250 PMV cryomicrotome at −20°C. Sections of 50 μm thickness were taken parallel to the oral and aboral surface at intervals of 1 mm and picked up with transparent adhesive tape. For histological observations, sections were fixed in a 4% formaldehyde solution in 100% ethanol for 1 min at −20°C and stained with hematoxylin-eosin. For autoradiography, sections were freeze dried inside the cryostat, sprayed lightly with talcum powder, and placed on X-ray film (Agfa-Gevaert Structurix D-10). After exposure at room temperature for two weeks, contact prints of the developed negatives were made using one standard exposure time and paper quality to allow direct comparison of results. Controls for chemography were satisfactory.

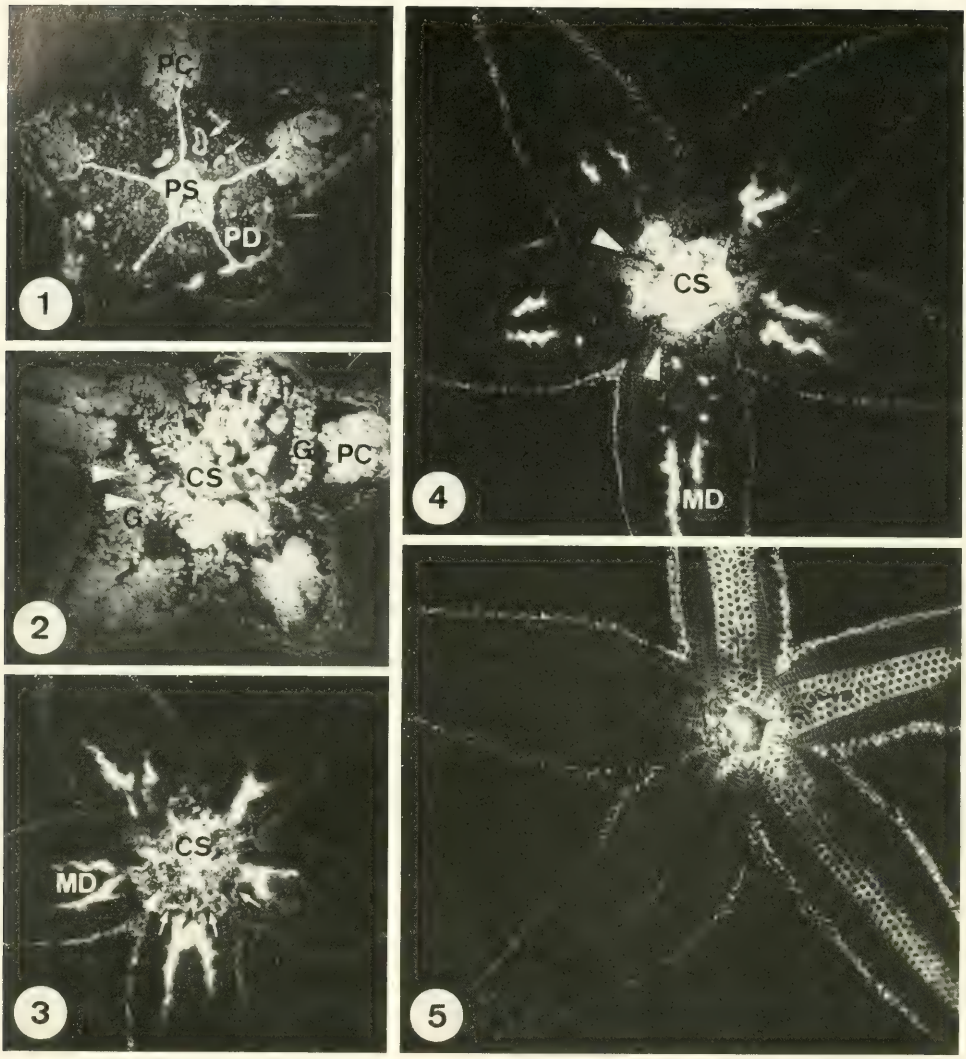
For microautoradiography, semi-thin sections (1 μm) of plastic embedded tissue were cut on a Reichert OMU 3 ultramicrotome. Sections were attached to gelatin coated slides by heating. Since pilot experiments revealed strong negative chemography, sections were stained first with Methylene-Blue/Azure II; slides were then coated by double immersion in 0.5% collodion in amyl acetate, and air dried for two days. The slides were dipped in Ilford K2 liquid nuclear emulsion (diluted 1:1 with 1% glycerol in distilled water), air dried, sealed in light-tight boxes containing a dessicant, and exposed at 5°C for 40 and 100 days. The micrographs presented in the next sections are made from preparations developed after 100 days.

For each incubation time and precursor used, one animal (sometimes two) was studied throughout from aboral to oral body wall by whole-body autoradiography, while gonad tissue from two to four animals (of both sexes when possible) was used for microautoradiography.

Results

Whole-body autoradiography after administration of radiolabeled leucine.

Digestive system. At all incubation times employed, the most prominent levels of tracer observed in whole-body sections are present in the walls of the cardiac and pyloric parts of the stomach and in the median ducts of the pyloric caeca (Figs. 1–10). The side lobes of the pyloric caeca reveal significant labeling only after short



FIGURES 1–10. Full-sized contact-autoradiographs of (parts of) whole-body sections (50 μm thickness) of specimens of *Asterias rubens*, frozen at timed intervals after oral administration of L-[^{14}C(U)]-leucine (0.37 MBq = 10 μCi per animal).

FIGURE 1. Animal sacrificed after one and a half hours. The roof of the pyloric stomach (PS), the five pyloric ducts (PD), and the walls of the rectal caeca (arrows) are distinctly labeled. Note diffuse labeling of the central region of the perivisceral coelomic cavity surrounding the stomach. PC: pyloric caeca.

FIGURE 2. Same animal as in Figure 1, section taken more orally. The folds of the cardiac stomach (CS) and the proximal parts of the pyloric caeca (PC) are strongly labeled. Gonads (G) are visible as highly lobulated strands of tissue and display radioactivity predominantly in regions close to the stomach (arrowheads).

FIGURE 3. Animal sacrificed after four hours. Cardiac stomach (CS) and median ducts (MD) of pyloric caeca are strongly labeled. Note diffuse radioactivity of the central region of the perivisceral coelomic cavity and narrow bands of tracer along the proximal parts of the peritoneal lining of the pyloric caeca (arrows).

FIGURE 4. Same animal as in Figure 3, section taken more orally to show radioactivity in cardiac stomach (CS) and median ducts (MD) of pyloric caeca. Note labeling of lobulated gonad tissue in regions close to the stomach (arrowheads).

FIGURE 5. Same animal as in Figure 3, section of ambulacral region. The body wall, podia (visible in cross-section as grouped circles), and mouth region are labeled.

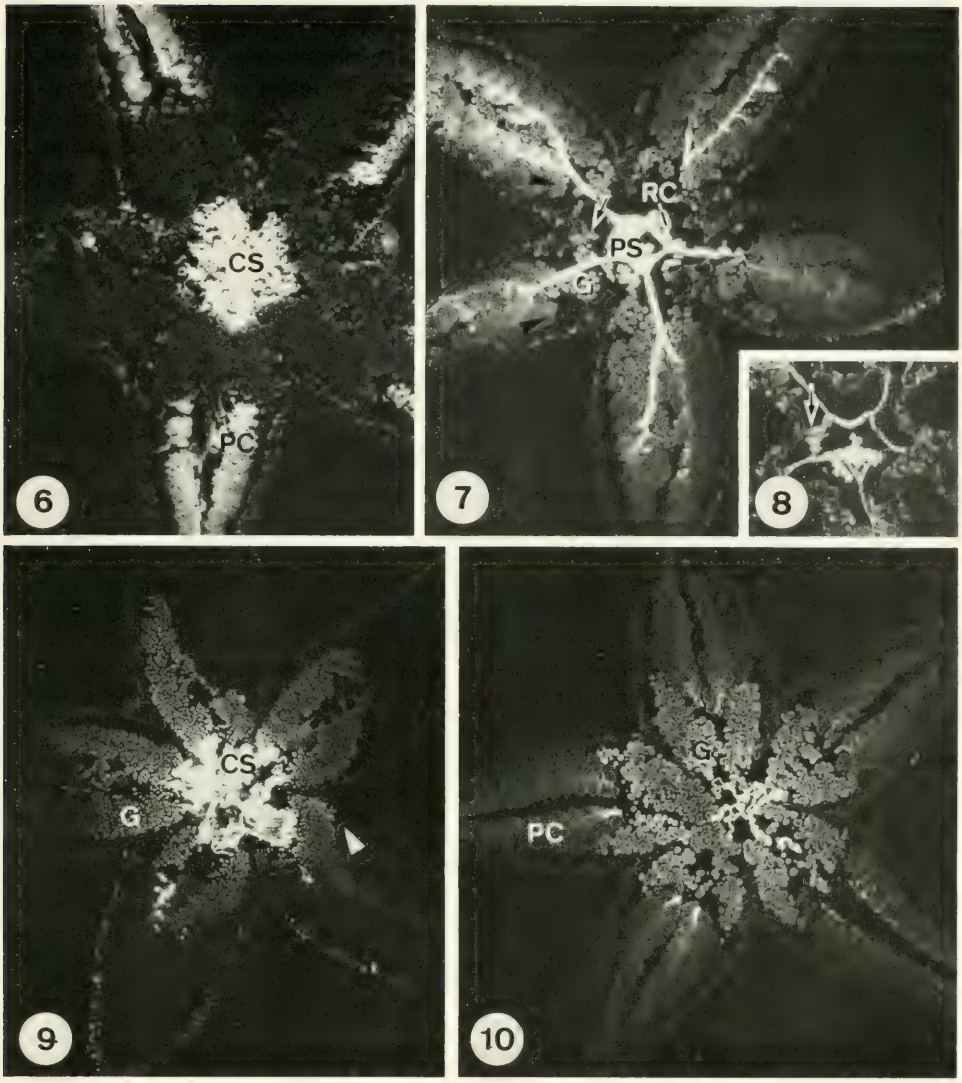


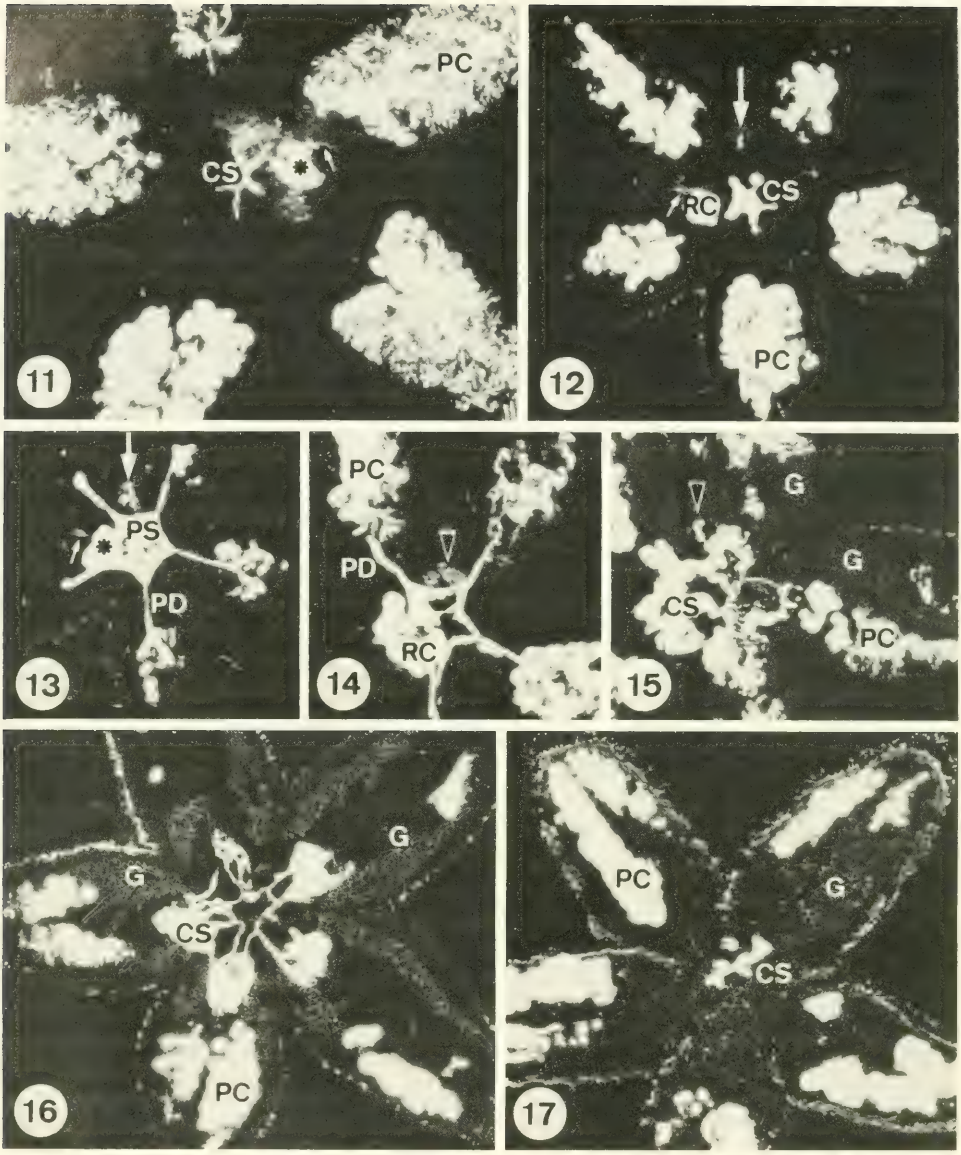
FIGURE 6. Animal sacrificed after six hours. Folds of cardiac stomach (CS) and side lobes of the pyloric caeca (PC) are labeled. The gonad tissue, present throughout the central area of the section, shows peripheral labeling.

FIGURE 7. Animal sacrificed after one day. The tissues of the pyloric stomach (PS), pyloric and median ducts (visible as broad white bands), and rectal caeca (RC) are strongly labeled. Moderate levels of radioactivity are present in the gonads (G). The side lobes of the pyloric caeca are slightly labeled. Note distinct narrow bands of radioactivity over the peritoneal lining of the pyloric caeca (black arrowheads). Arrow points to slightly labeled axial organ.

FIGURE 8. Same animal as in Figure 7, section taken more aborally to show labeling of gastric hemal tufts (arrow).

FIGURE 9. Animal sacrificed after one day (other animal as in Figs. 7, 8). The folds of the cardiac stomach (CS) are strongly labeled. Moderate levels of radioactivity are present in the gonads (G) with highest levels near the stomach. White arrowhead indicates labeling of peritoneal lining of pyloric caeca; side lobes are labeled only very little. Labeling of the walls of the ampullae can be observed in the upper and upper right arm.

FIGURE 10. Animal sacrificed after sixteen days. Folds of cardiac stomach (center) and gonads (G) are distinctly labeled. PC: pyloric caeca.



FIGURES 11–17. Full-sized contact autoradiographs of (parts of) whole-body sections (50 μm thickness) of specimens of *Asterias rubens*, frozen at timed intervals after oral administration of [^{14}C(U)]-palmitic acid (0.37 MBq = 10 μCi per animal).

FIGURE 11. Animal sacrificed after one and a half hours. The cardiac stomach (CS) and pyloric caeca (PC) are strongly labeled. Arrow indicates narrow band of radioactivity along the lining of a gonad. The central area of the coelomic activity is distinctly labeled (asterisk).

FIGURE 12. Animal sacrificed after six hours. High levels of radioactivity are present in cardiac stomach (CS), rectal caecum (RC), and pyloric caeca. Small arrow indicates tiny band of radioactivity in interbrachial septum; big arrow points to strongly labeled axial organ.

FIGURE 13. Same animal as in Figure 12, section taken more aborally. Note strong labeling of the roof of the pyloric stomach (PS), pyloric ducts (PD), and rectal caecum (asterisk). Small arrow indicates band of tracer in the interbrachial septum; big arrow indicates labeled gastric hemal tufts.

FIGURE 14. Animal sacrificed after one day. High levels of tracer are present in pyloric caeca (PC), pyloric ducts (PD), and rectal caeca (RC). Arrowhead indicates moderately labeled gastric hemal tufts.

FIGURE 15. Same animal as in Figure 14, section taken more orally to show labeling of axial organ (arrowhead) and folded cardiac stomach (CS). Gonads (G) are slightly labeled. PC: pyloric caeca.

periods of incubation (Figs. 2, 6); generally, levels of radioactivity in the side lobes are relatively low and do not noticeably increase during prolonged incubation (Figs. 7, 9, 10). At the site of the coelomic epithelium of the pyloric caeca, a sharp band of radioactivity is present after incubation times of four hours and more (Figs. 3, 7, 9). Shortly after feeding, this band is especially conspicuous in regions of the pyloric caeca that lie close to the stomach (Fig. 3).

The walls of the rectal caeca reveal significant amounts of tracer in all sections studied (*e.g.*, Figs. 1, 7).

Coelomic cavity and hemal tissues. Shortly after ingestion of labeled leucine, scattered labeling of the coelomic area surrounding the pyloric stomach is observed (Figs. 1, 3). The gastric hemal tufts are moderately labeled after one day (Fig. 8); radioactivity of these organs is less pronounced after prolonged incubation. Whereas some moderate labeling of the axial organ has been observed after one day (Fig. 7), distinct bands of radioactivity indicating the presence of tracer within the aboral ring have not been detected.

Gonads and body wall. Labeling of the gonads one and a half hours after ingestion is especially manifest in regions close to the stomach (Figs. 2, 4). After four hours, the gonads become labeled more intensely and more uniformly—although radioactivity remains localized mainly in the periphery of the gonad tissue—and ultimately may display levels of radioactivity higher than those observed in the side lobes of the pyloric caeca (Figs. 7, 9, 10).

The body wall is labeled relatively little at all incubation times. High levels of tracer are present in the ambulacral tissues and tube feet (Fig. 5); these levels increase during prolonged incubation.

Whole-body autoradiography after ingestion of radiolabeled palmitic acid

Digestive system. One and a half hours after ingestion, tracer is seen dispersed throughout the pyloric caeca (Fig. 11). After prolonged incubation, the most prominent levels of tracer observed in whole-body sections are present in the pyloric caeca. At all incubation times, very pronounced levels of tracer are also present in the walls of the pyloric and cardiac parts of the stomach (Figs. 11–17).

The walls of the rectal caeca remain distinctly labeled until 16 days after administration. The rectal lumen is also strongly labeled after shorter periods of incubation (Figs. 12, 13).

Coelomic cavity and hemal organs. One and a half hours after ingestion, conspicuous amounts of tracer are present in the central area of the coelomic cavity near the cardiac and pyloric parts of the stomach (Fig. 11). The gastric hemal tufts are distinctly labeled after 6 to 24 hours (Figs. 13, 14). During prolonged incubation, the amounts of tracer present in the gastric hemal tufts seem to decrease; the same applies to the axial organ (Figs. 12, 15). Six hours after ingestion, distinct bands of radioactivity along the interbrachial septum indicate labeling of the aboral ring (Figs. 12, 13).

Gonads and body wall. After one and a half hours, distinct narrow bands of label can be distinguished along the margins of the gonads in regions close to the stomach (Fig. 11). After six hours to one day, gonads are slightly labeled (Fig. 15). Levels of

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FIGURE 16. Animal sacrificed after four days. Highest levels of radioactivity are present in pyloric caeca (PC) and walls of cardiac stomach (CS). Sharp bands of radioactivity are observed over the lining of the gonads (G).

FIGURE 17. Animal sacrificed after sixteen days. Note strong labeling of the pyloric caeca (PC), cardiac stomach (CS), and body wall. The periphery of the gonads (G) is sharply labeled.

radioactivity slightly increase during prolonged incubation but stay relatively low (Figs. 16, 17); label remains distinctly localized in the gonadal periphery.

The body wall is slightly labeled after all incubation times employed. The tube feet and ambulacral tissues display relatively low levels of tracer after incubation times of six hours and longer.

Microautoradiography of gonad tissue

Only very low levels of label from ^{14}C-leucine are observed in semi-thin sections of ovaries. Figure 19 shows labeling of the ooplasm of fully vitellogenic oocytes six hours after ingestion of leucine; regions of the ooplasm near the gonad wall reveal highest levels of radioactivity. Little radioactivity is present in the gonad wall. In sections of ovaries fixed after other incubation times, levels of radioactivity are too low to be discernable from background activity.

In testes, label from leucine is generally much easier to observe. Figure 18 (arrows) shows small patches of silver grains above the myoepithelial lining of the genital coelomic sinus one and a half hour after ingestion of leucine; no radioactivity is present in spermatogenic columns, genital hemal sinus, or wall of this testis. After prolonged incubation, increasing levels of radioactivity are observed in the ground substance of the genital hemal sinus (Fig. 21), whereas the outer connective tissue layer and visceral peritoneum ("outer sac") are only slightly labeled (Figs. 20–24). Label becomes incorporated in basal regions of the spermatogenic columns initially (Fig. 20) and spreads throughout the columns during prolonged incubation (Figs. 21, 22); after 16 days, the chains of spermatids at the tip of the columns are also labeled (Fig. 23).

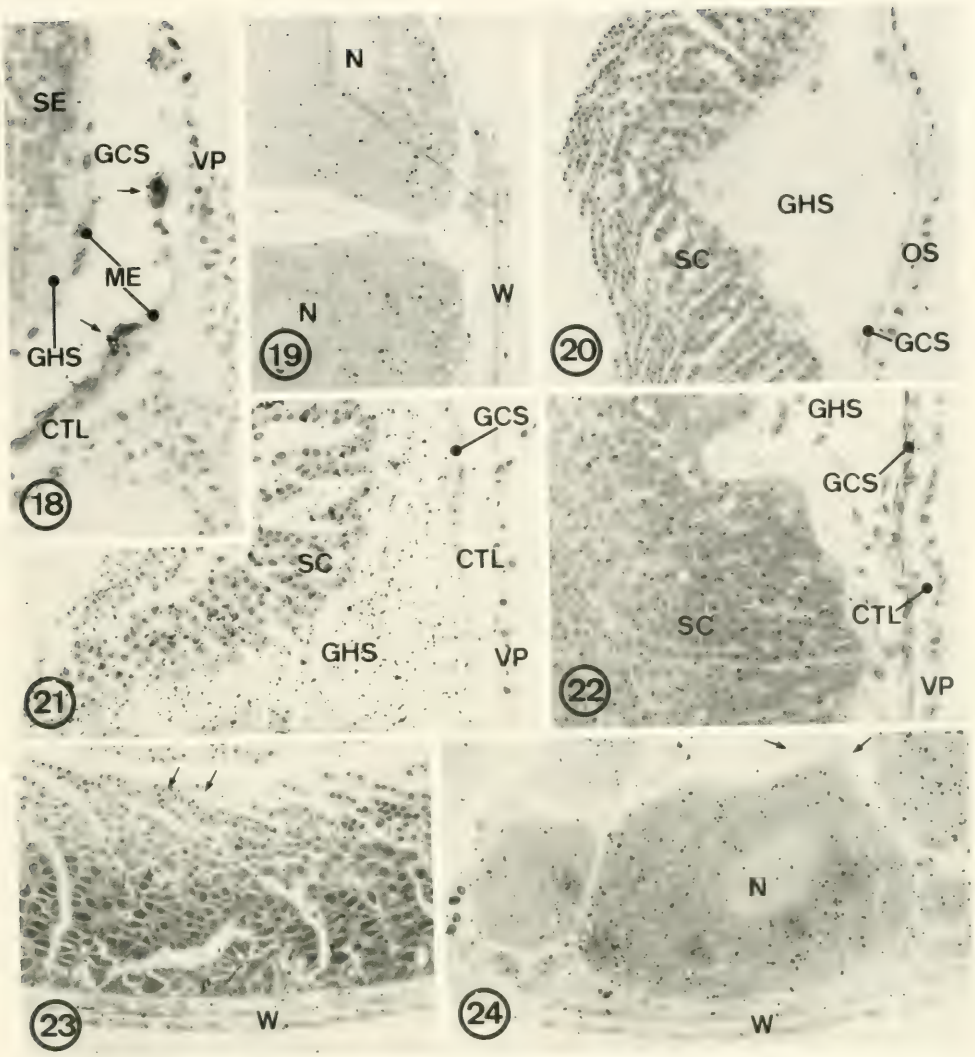
After administration of radiolabeled palmitic acid, no radioactivity clearly discernable from background levels is observed in testes and ovaries after incubation times of one and a half hours to one day. For longer periods of incubation, only sections of ovaries were available; in these sections, distinct labeling was observed not earlier than 16 days after ingestion, with highest levels of radioactivity present throughout the ooplasm and little or no radioactivity in the gonad wall (Fig. 24). No distinct labeling of follicle cells (Fig. 24, arrows) or coelomocytes, frequently attached to the peritoneum, has been observed.

Discussion

Source organs: digestive system

The observed distribution of radiolabel in "source" organs (stomach and pyloric caeca), "transport" organs (hemal tissues and coelomic cavities) and "recipient" organs (gonads, ambulacral tissues, and body wall) reveals several interesting features which demand comparison with earlier autoradiographic and analytical studies.

Little radioactivity could be recovered in isolated pyloric caeca, ovaries, coelomic fluid, and coelomocytes of *Asterias rubens* after oral administration of radiolabeled trioleylglycerol, leucine, and glucose (Oudejans and Rutten, 1982; Oudejans *et al.*, 1983). This is in agreement with the present observations revealing high levels of radioactivity—especially after administration of leucine—predominantly present in the tissues of the pyloric and cardiac parts of the stomach. To a lesser degree, the same holds for palmitic acid although label from this compound is distributed throughout the pyloric caeca much easier than the label from leucine; apparently, absorption of dietary fatty acid by the major storage organs of *Asterias rubens* is a fast process.



FIGURES 18–24. Stained autoradiographs of 1 μm sections of glutaraldehyde/osmium tetroxide-fixed gonad tissue of specimens of *Asterias rubens*, dissected at timed intervals after oral administration of L-[^{14}C(U)]-leucine or [^{14}C(U)]-palmitic acid (0.37 MBq = 10 μCi per animal). All magnifications 350×.

FIGURE 18. Testis, one and a half hours after ingestion of leucine. Arrows indicate labeling of the outer myoepithelial lining (ME) of the genital coelomic sinus (GCS). SE: spermatogenic epithelium; GHS: genital hemal sinus; CTL: connective tissue layer of outer sac; VP: visceral peritoneum.

FIGURE 19. Ovary, six hours after ingestion of leucine. Two vitellogenic oocytes show radioactivity, predominantly near the wall (W) of the ovary. N: nuclei of oocytes.

FIGURE 20. Testis, six hours after ingestion of leucine. Some radiolabel is present in basal regions of the spermatogenic columns (SC). Little or no tracer is present in the outer sac (OS) or in the swollen genital hemal sinus (GHS) and the amoeboid cells contained. GCS: compressed genital coelomic sinus.

FIGURE 21. Testis, one day after ingestion of leucine. Prominent levels of tracer are present throughout the spermatogenic columns (SC) and the ground substance of the genital hemal sinus (GSH). GCS: compressed genital coelomic sinus; CTL: connective tissue layer of outer sac; VP: visceral peritoneum.

FIGURE 22. Testis, four days after ingestion of leucine. Label is present throughout the spermatogenic columns (SC). GHS: genital hemal sinus with amoeboid cells; GCS: compressed genital coelomic sinus with myoepithelial lining; CTL: connective tissue layer of outer sac; VP: visceral peritoneum.

FIGURE 23. Testis, sixteen days after ingestion of leucine. Label, which is present mainly in basal regions of the spermatogenic columns, can also be observed in the wall (W) and in spermatids (arrows) at the tips of the columns.

FIGURE 24. Ovary, sixteen days after ingestion of palmitic acid. Distinct levels of radioactivity are present in the ooplasm of vitellogenic oocytes. N: oocyte nucleus; W: wall of ovary. Arrows indicate follicle cells.

Autoradiographs of paraffin sections of *Echinaster graminicolus* fed labeled amino acids (Ferguson, 1984) also revealed high levels of radioactivity in cardiac stomach and pyloric caeca, indicating substantial incorporation of amino acid into insoluble proteinoid material in these organs. Such incorporation was also demonstrated biochemically for the pyloric caeca of *Asterias rubens* (Oudejans *et al.*, 1983). Broertjes *et al.* (1980b) studied autoradiographs of the strongly folded roof of the pyloric stomach of the same species after oral administration of radiolabeled leucine and observed a progression of relatively low levels—as compared with the results from the present study—of insoluble radiolabel from apical regions of the digestive epithelium into deeper layers of the pyloric stomach tissue. Little radioactivity was observed in paraffin sections of the pyloric stomach of *Echinaster* fed radiolabeled amino acids (Ferguson, 1984). In contrast with these two studies, the present investigation reveals high levels of radioactivity in frozen sections of the roof and side walls of the pyloric stomach after administration of leucine, suggesting that amino acids, absorbed in these regions, are relatively little incorporated into tissue-protein—as demonstrated by paraffin section autoradiography—and may therefore be transferred directly to the adjacent hemal tissues (gastric hemal tufts) or coelomic fluid.

Using γ-camera equipment, Broertjes *et al.* (1982) could follow the fate of ingested ^{125}I-labeled mussel protein *in vivo*. Results of these experiments lent support to a model postulated earlier (Broertjes *et al.*, 1980a, b). According to this model, materials stored temporarily in the pyloric caeca are released into the caecal lumen and moved by mucoid-ciliary action into the direction of the folded roof of the pyloric stomach where they are absorbed by the digestive epithelium and pass through to the gastric hemal tufts. The present study does not give evidence for the release of stored nutrients into the caecal lumen; according to Broertjes *et al.* (1982) it would however take about one month after initial storage of radiolabeled food before such release occurs.

Distinct labeling of the coelomic lining of the pyloric caeca shortly after ingestion of radiolabeled leucine demonstrates that the coelomic epithelium absorbs nutrients from the coelomic fluid as was demonstrated also *in vitro* by Ferguson (1964b, 1968). The observed phenomenon does not necessarily prove net uptake of amino acid from the coelomic fluid; simultaneously with the observed uptake of labeled material, a more substantial release—due to the steep concentration gradient—may occur (Ferguson, 1982) which can not be monitored inasmuch as it concerns unlabeled compounds from already existing caecal depots. Up to now, such release has only been demonstrated using pyloric caeca *in vitro* submersed in nutrient depleted sea water (Ferguson, 1964b).

The observed high levels of tracer in the rectal caeca are in agreement with information from morphological studies indicating a high power of absorption of these organs (Jangoux, 1972, 1976).

Transport organs: coelomic cavity and hemal tissues

Shortly after ingestion of labeled leucine or palmitic acid, substantial amounts of tracer are present in the central area of the perivisceral coelomic cavity near the stomach. This unique observation, accomplished by the special features of the technique employed, conforms to the results of earlier studies (Ferguson, 1964a; Oudejans *et al.*, 1983) which show the rapid exchange of molecules between digestive system and coelomic fluid. Stained whole-body sections show eosinophilia in these coelomic regions, indicating the presence of coelomocytes. These amoeboid cells probably function in some transfer of fatty acids as it is unlikely that substantial amounts of these compounds are dissolved in the coelomic fluid. The high specific radioactivity of coelomocyte lipids, observed after oral administration of labeled trioleylglycerol, may support this view (Oudejans and Rutten, 1982).

Although initial distribution of ingested nutrients through the coelomic fluid and coelomocytes—also indicated by the rapid labeling along the peritoneal lining of gonads and pyloric caeca in regions close to the stomach—seems evident from the results presented here, the turnover rates of coelomic fluid compounds are by far insufficient to meet the demands of the developing ovaries during vitellogenesis (Beijnink *et al.*, 1984a). Alternatively, several authors have suggested involvement of the hemal system in transfer of materials from digestive organs to gonads and other areas of the body (reviews: Ferguson, 1982; Walker, 1979, 1982). Autoradiographic data, interpreted in favor of this hypothesis, were given by Ferguson (1970) who injected radiolabeled amino acids into the coelomic cavity of *Echinaster* and observed a marked accumulation of tracer in the radial strands of hemal tissue of the ambulacral regions. Significantly, label appeared simultaneously at different sites throughout the hemal strands; no progression of label from possible sites of origin towards sites of utilization was observed. Recently, Ferguson (1984) published an autoradiographic study indicating a progressive incorporation—from disc to gonads—of radiolabel derived from ingested amino acids into the ground substance of the aboral hemal ring and of the genital hemal sinuses. Again, it should be emphasized that these studies primarily demonstrate the incorporation of amino acid into insoluble material; thus, they are more indicative of synthesis or deposition of proteinoid material within hemal tissue rather than of translocation of nutrients through hemal tissue. In fact, it is unlikely that displacement of the mucoid hemal ground substance through the diminuitive hemal sinuses could occur at rates high enough to account for the prominent levels of radioactivity observed in the genital hemal sinuses 24 hours after ingestion of labeled leucine (Fig. 21). Alternatively, the hemal tissues might function as storage reservoirs within the surrounding perihemal coelomic sinuses which would then be primarily responsible for translocation; circulation within these fluid-filled coelomic channels is easily maintained by movement of the flagella of the coelomic lining (Walker, 1979). Such a storage function of hemal tissue was proposed earlier for species of three echinoderm classes (Grimmer and Holland, 1979; Walker, 1979; Ferguson, 1984; Jackson and Fontaine, 1984).

Up to now, no autoradiographic experiments designed to elucidate pathways of translocation of water-insoluble lipid compounds have been performed on echinoderm species. This study provides some evidence for the involvement of hemal tissues or enclosing coelomic sinuses in translocation of fatty acids: A very pronounced labeling of the gastric hemal tufts, the axial organ, and, presumably, the aboral ring is observed shortly after ingestion of palmitic acid. The subsequent decrease of levels of radioactivity in these hemal organs prior to the increase of radioactivity in the gonads may suggest the displacement of radiolabel through hemal tissue, or more likely, as argued above, the progressive temporary incorporation of label into hemal tissue from the surrounding (perihemal) coelomic cavities prior to release in the opposite direction.

Recipient organs: body wall and gonads

The body wall and associated ambulacral tissues show distinct levels of radioactivity shortly after ingestion of labeled leucine and, to a much lesser degree, of palmitic acid. Although several changes of fresh sea water have been employed during initial incubation, significant amounts of labeled compounds may have been present in the sea water due to leakage of precursor out of the esophagus. Therefore, the observed radioactivity of the body wall may be partly the result of epidermal uptake, a process well established in echinoderms (review: Bamford, 1982). As far as labeling of the body wall results from internal supplies, translocation through the coelomic fluid is probably involved.

Gonads display prominent levels of radioactivity at relatively short intervals after administration of labeled leucine. Biochemical analysis (Oudejans *et al.*, 1983) revealed the labeling of proteins and amino acids in the ovaries of *Asterias rubens* to be about equal one day after ingestion of labeled leucine. Microautoradiography also shows a substantial incorporation of label from leucine into insoluble (proteinoid) material in both testes and ovaries. Radiolabel derived from palmitic acid is conveyed apparently much more slowly to the gonads. While label from ingested trioleylglycerol after short periods of incubation (one hour to one day) could be recovered primarily in the triacylglycerol fraction after thin-layer chromatography of total lipids from sea star ovaries (Oudejans and Rutten, 1982), microautoradiography (Fig. 24) reveals that label from fatty acid is incorporated into compounds of the ooplasm that are not lost during dehydration in ethanol and propylene oxide [*e.g.* (glyco-)lipoproteins].

In the previous paragraphs, we have discussed the evidence for translocation either through the perivisceral coelomic cavity or through the (peri-)hemal system. As mentioned above, whole-body autoradiographs with gonad tissue show labeling patterns primarily supporting the first possibility; also, autoradiographic studies have demonstrated the penetration of thymidine, injected intra-coelomically (Walker, 1980; Van der Plas *et al.*, 1983) and adenine, applied *in vitro* (Toole *et al.*, 1974), into deeper layers of asteroid gonads. No patterns suggesting the dispersion of labeled substances throughout the gonads, away from the points of attachment to the body wall and the genital branches of the aboral ring, have been observed in whole-body autoradiographs. Analysis of the microautoradiographs, however, leads to other conclusions with regard to routes of translocation: remarkably, no label from leucine is incorporated into tissues of the outer sac of the gonad wall in sections where substantial amounts of tracer are observed in the germinal cells and/or the genital hemal sinus. This implies that nutrients such as leucine either pass from the coelomic fluid through the outer sac without being incorporated into tissue proteins, or more likely reach the inner sac by translocation through the genital coelomic and hemal sinuses. If one accepts the improbability of rapid translocation through the hemal sinuses, the coelomic sinuses of the aboral ring and gonad wall thus may perform crucial functions in short term nutrient transport towards the gonads. This view seems to be supported by the observed labeling of the myoepithelial lining of the genital coelomic sinus one and a half hours after ingestion of labeled leucine (Fig. 18, arrows). Of course, radiolabel within this fluid-filled sinus cannot be demonstrated with the microautoradiographic technique employed.

This study demonstrates a substantial incorporation of label from leucine and—to a lesser degree and only after prolonged incubation—from palmitic acid into the ground substance of the genital hemal sinus. Similar observations were made recently by Ferguson (1984) who demonstrated labeling of the genital hemal sinuses in spawned gonads of *Echinaster* fed radiolabeled amino acids. Apparently, both in true vitellogenic or spermatogenic stages, as studied in the present investigation, and in agametogenic stages, as studied by Ferguson (1984), incorporation of materials into hemal ground substance occurs. Histological and ultrastructural studies (Schoenmakers *et al.*, 1981; Beijnink *et al.*, 1984b) indicate that components of this ground substance are conveyed ultimately to vitellogenic oocytes; a recent immunocytochemical study demonstrates a close relationship between yolk glycolipoprotein and hemal ground substance (Beijnink *et al.*, 1984c).

Inasmuch as hemal sinuses are not recipient organs but transport organs, prominent levels of tracer within these sinuses point to high turnover rates, and, thus, to high rates of utilization of hemal ground substance. How incorporation of label from amino acids into proteinoid components of the virtually acellular hemal ground

substance (*e.g.*, Fig. 21) can be achieved, is only one of several intriguing problems that remain to be solved in order to better understand the complex mechanisms of nutrient digestion, translocation, and utilization in echinoderms.

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LITERATURE CITED

BAMFORD, D. 1982. Epithelial absorption. Pp. 317–330 in *Echinoderm Nutrition,* M. Jangoux and J. M. Lawrence, eds. Balkema, Rotterdam.

BEIJNINK, F. B., I. VAN DER SLUIS, AND P. A. VOOGT. 1984a. Turnover rates of fatty acid and amino acid in the coelomic fluid of the sea star *Asterias rubens:* Implications for the route of nutrient translocation during vitellogenesis. *Comp. Biochem. Physiol.* **78B:** 761–767.

BEIJNINK, F. B., C. W. WALKER, AND P. A. VOOGT. 1984b. An ultrastructural study of relationships between the ovarian haemal system, follicle cells, and primary oocytes in the sea star, *Asterias rubens:* Implications for oocyte nutrition. *Cell Tissue Res.* **238:** 339–347.

BEIJNINK, F. B., J. J. S. BROERTJES, F. BRANDS, AND P. A. VOOGT. 1984c. Immunocytochemical demonstration of vitellogenic substances in the haemal system of the sea star, *Asterias rubens. Mar. Biol. Lett.* **5:** 303–313.

BROERTJES, J. J. S., AND G. POSTHUMA. 1978. Direct visualization of the haemal system in starfish by a staining procedure. *Experientia* **34:** 1243–1244.

BROERTJES, J. J. S., G. POSTHUMA, P. DEN BREEJEN, AND P. A. VOOGT. 1980a. Evidence for an alternative transport route for the use of vitellogenesis in the sea-star *Asterias rubens* (L.). *J. Mar. Biol. Assoc. U. K.* **60:** 157–162.

BROERTJES, J. J. S., G. POSTHUMA, F. B. BEIJNINK, AND P. A. VOOGT. 1980b. The admission of nutrients from the digestive system into the haemal channels in the sea-star *Asterias rubens* (L.). *J. Mar. Biol. Assoc. U. K.* **60:** 883–890.

BROERTJES, J. J. S., J. N. JENS, D. VAN OUDHEUSDEN, M. DE BRUIN, AND P. A. VOOGT. 1982. Demonstration of nutrient flow in the starfish *Asterias rubens* (L.) with ^{125}I labelled proteins. *Neth. J. Zool.* **32:** 472–478.

FERGUSON, J. C. 1963. An autoradiographic study of the distribution of ingested nutrients in the starfish, *Asterias forbesi. Am. Zool.* **3:** 524.

FERGUSON, J. C. 1964a. Nutrient transport in starfish. I. Properties of the coelomic fluid. *Biol. Bull.* **126:** 33–53.

FERGUSON, J. C. 1964b. Nutrient transport in starfish. II. Uptake of nutrients by isolated organs. *Biol. Bull.* **126:** 391–406.

FERGUSON, J. C. 1968. Transport of amino acids by starfish digestive glands. *Comp. Biochem. Physiol.* **24:** 921–931.

FERGUSON, J. C. 1970. An autoradiographic study of the translocation and utilization of amino acids by starfish. *Biol. Bull.* **138:** 14–25.

FERGUSON, J. C. 1982. Nutrient translocation. Pp. 373–393 in *Echinoderm Nutrition,* M. Jangoux and J. M. Lawrence, eds. Balkema, Rotterdam.

FERGUSON, J. C. 1984. Translocative functions of the enigmatic organs of starfish—the axial organ, hemal vessels, Tiedemann's bodies, and rectal caeca: An autoradiographic study. *Biol. Bull.* **166:** 140–155.

GRIMMER, J. C., AND N. D. HOLLAND. 1979. Haemal and coelomic circulatory systems in the arms and pinnules of *Florometra serratissima* (Echinodermata: Crinoidea). *Zoomorphologie* **94:** 93–109.

JACKSON, L. E., AND A. R. FONTAINE. 1984. Nutrient and nitrogenous waste concentrations in the fluid transport systems of a holothurian. *Comp. Biochem. Physiol.* **78A:** 123–128.

JANGOUX, M. 1972. La structure fine des caecums rectaux de deux Asteriidae, *Marthasterias glacialis* (L.) et *Coscinasterias tenuispina* (Lam.). *Z. Zellforsch.* **128:** 366–384.

JANGOUX, M. 1976. Fonctions des caecums rectaux chez l'étoile de mer, *Asterias rubens* L. *Thalassia Jugoslav.* **12:** 181–186.

OUDEJANS, R. C. H. M., AND V. P. G. M. RUTTEN. 1982. Fate of the glycerol and acyl moieties from dietary trioleylglycerol in the sea star, *Asterias rubens. Comp. Biochem. Physiol.* **73B:** 685–693.

OUDEJANS, R. C. H. M., A. DE RUYTER, A. J. VAN DER PLAS, AND P. A. VOOGT. 1983. Fate of ingested leucine and glucose in the sea star, *Asterias rubens,* during its annual reproductive cycle. *Comp. Biochem. Physiol.* **76B:** 591–598.

SCHOENMAKERS, H. J. N., P. H. J. M. COLENBRANDER, J. PEUTE, AND P. G. W. J. VAN OORDT. 1981. The anatomy of the ovaries of the starfish *Asterias rubens* (Echinodermata). A histological and ultrastructural study. *Cell Tissue Res.* **217:** 577–597.

TOOLE, B., A. W. SCHUETZ, and E. BOYLAN. 1974. Uptake and cellular localization of ^{3}H-1-methyladenine and ^{3}H-adenine in the starfish gonad and oocytes. *Gen. Comp. Endocrinol.* **22:** 199–208.

VAN DER PLAS, A. J., F. BRANDS, AND P. A. VOOGT. 1983. An autoradiographic investigation of the proliferative activity of pyloric caeca and gonads of *Asterias rubens. J. Morphol.* **177:** 51–58.

WALKER, C. W. 1979. Ultrastructure of the somatic portion of the gonads in asteroids, with emphasis on flagellated collar cells and nutrient transport. *J. Morphol.* **162:** 127–162.

WALKER, C. W. 1980. Spermatogenic columns, somatic cells, and the microenvironment of germinal cells in the testes of asteroids. *J. Morphol.* **166:** 81–107.

WALKER, C. W. 1982. Nutrition of gametes. Pp. 449–468 in *Echinoderm Nutrition,* M. Jangoux and J. M. Lawrence, eds. Balkema, Rotterdam.

Reference: *Biol. Bull.* **167:** 683–697. (December, 1984)

PHOTOBIOLOGY OF THE SYMBIOTIC SEA ANEMONE, *ANTHOPLEURA ELEGANTISSIMA:* DEFENSES AGAINST PHOTODYNAMIC EFFECTS, AND SEASONAL PHOTOACCLIMATIZATION

JAMES A. DYKENS[1] AND J. MALCOLM SHICK

Department of Zoology, University of Maine Orono, Maine 04469

ABSTRACT

The sea anemone *Anthopleura elegantissima,* which contains photosynthetic symbionts (zooxanthellae), responds both biochemically and behaviorally to the combined environmental stresses of exposure to sunlight and photosynthetically generated hyperbaric O_2. Activities of the enzymes superoxide dismutase (SOD) and catalase, which act in concert as defenses against oxygen toxicity, parallel the distribution of chlorophyll. *A. elegantissima* shows a finely controlled contraction behavior which shades the zooxanthellae and reduces O_2 production, but which leaves the body column tissues directly exposed to sunlight. However, the body column contains disproportionately high SOD and catalase activities as defenses against photodynamic damage. This additional role of SOD is demonstrated by shade-adapted aposymbiotic anemones in which SOD and catalase activities increase by 590% and 100% respectively following a 7 day exposure to sunlight. In response to elevated levels of O_2 and sunlight exposure, *A. elegantissima* attaches gravel and other debris to its body surface which serves as a sunscreen that effectively reduces zooxanthella expulsion during exposure to bright sunlight. Finally, anemone chlorophyll content fluctuates on a seasonal basis, varying inversely with mean solar radiation. These seasonal changes are not due to corresponding changes in the number of algal cells, but rather to changes in the chlorophyll content and chlorophyll $a{:}c_2$ ratio of a fairly uniform standing crop of zooxanthellae.

INTRODUCTION

Much of the recent interest in plant-animal symbioses has focused on nutritionally beneficial aspects of these associations. Mutually advantageous exchanges of organic and inorganic metabolites between photosynthetic endosymbiont and host animal have been demonstrated (Trench, 1979; Muscatine, 1980; Cook, 1983) and attempts to quantify the contributions made by photosynthetically fixed carbon to the host's metabolic needs have been progressively refined (McCloskey *et al.,* 1978; Muscatine *et al.,* 1981; Muscatine *et al.,* 1984). There are, however, a number of potentially deleterious interactions between the host and symbiont that are unavoidable consequences of the nature of these symbioses, namely the toxicity of molecular oxygen produced as an obligatory by-product of photosynthesis and the requirement for sunlight exposure and the associated potential for photodynamic damage.

Although the molecular bases for oxygen toxicity are not generally agreed upon (Halliwell, 1978, 1982; Fee, 1980; Sullivan *et al.,* 1983), it is evident that because of

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[1] Present address for reprint requests: Department of Pharmacology, New York University Medical Center, 550 First Avenue, New York, New York 10016.

orbital spin restrictions molecular oxygen preferentially undergoes univalent reductions which, directly or indirectly, can generate the superoxide radical (O_2^-), the hydroxyl radical ($OH^\bullet$) and hydrogen peroxide (H_2O_2) (Halliwell, 1979; Fridovich, 1977a, b, 1978; Cadet and Teoule, 1978; Hill, 1978), three of the most reactive chemical species in biological systems. The enzymes superoxide dismutase (SOD) and catalase act in concert to minimize the toxicity of oxygen by removing superoxide radicals and H_2O_2, thereby preventing the formation of hydroxyl radicals (McCord and Fridovich, 1969; Fridovich, 1978; Steinman, 1982; Chapman and Schopf, 1983). In addition to ultraviolet (UV) wavelengths of sunlight below 380 nm, long recognized as damaging to biological materials (Clayton, 1977; Jokiel, 1980; Jokiel and York, 1982), wavelengths of visible light are also responsible for a variety of biologically disruptive reactions that are synergistically mediated by the presence of molecular oxygen (Clayton, 1977). These reactions, dependent on the presence of photosensitizing agents (*e.g.,* flavins, chlorophyll), include single electron transfers capable of generating superoxide radicals, as well as photochemical oxygenations and photoproduction of highly reactive singlet oxygen (Clayton, 1977; Khan, 1978; van Ginkel and Raison, 1980; Cooper and Zika, 1983).

The abundance of photosynthetically generated molecular oxygen and concurrent exposure to sunlight experienced by animals containing symbiotic algae present ideal conditions for biologically damaging interactions between sunlight and oxygen. This may be particularly true for sessile, soft-bodied marine invertebrates that occupy the intertidal region and are directly exposed to sunlight.

The West Coast intertidal sea anemone *Anthopleura elegantissima* (Brandt) harbors intracellular dinoflagellates (zooxanthellae) as endosymbionts and shows biochemical and behavioral adaptations to the environmental stresses of intense irradiance and simultaneous exposure to hyperbaric oxygen. Biochemical adaptations include maintaining levels of SOD and catalase activity in direct proportion to its algal complement (Dykens, 1984) as well as the presence of a UV absorbing pigment in anemones accustomed to sunlight exposure (Shick and Dykens, 1984). In addition to these biochemical defenses, *A. elegantissima* contracts during periods of peak irradiance (Pearse, 1974; Shick and Dykens, 1984) and attaches a cover of shells and other debris to its body column (Hart and Crowe, 1977; Dykens and Shick, 1983), both of which shade the zooxanthellae and reduce exposure to sunlight. However, acute exposure to intense sunlight causes *A. elegantissima* to expel large numbers of intact zooxanthellae (Pearse, 1974), a response also seen in other symbiotic sea anemones (Steele, 1976). Finally, unlike tropical symbiotic species, the temperate *A. elegantissima* experiences varying intensities of solar radiation that fluctuate seasonally by 450% even in central areas of its distribution (Barbour *et al.,* 1973). In this report we examine more fully the localization of anemone SOD and catalase and present evidence of an additional role for these enzymes as defenses against photodynamic damage. We also examine the effectiveness of attached debris as a sunscreen and present data on seasonal variations in numbers of zooxanthellae and chlorophyll content.

Materials and Methods

All specimens of *Anthopleura elegantissima* (Brandt) were collected from the intertidal region of the south jetty at the entrance of Bodega Harbor, California. Anemones were taken from several low intertidal clones at mean lower low water and from high intertidal clones 1.3 m above that level. Aposymbiotic anemones totally lacking symbiotic algae were collected from constantly shaded crevices along the jetty at a mid-intertidal height. Anemones were returned to Bodega Marine Laboratory

and placed in an outdoor flowing sea water system (14°C) in sunlight (for irradiance data see Shick and Dykens, 1984).

Enzyme determinations and localization

Freshly collected low-intertidal anemones were dissected into the tissues of the tentacles and oral disc, body column, and basal disc which were individually weighed and gently homogenized in sea water (Brinkmann Polytron homogenizer; 10% wt/vol). An aliquot of the crude tissue homogenate was removed for chlorophyll extraction prior to centrifugation at 900 × *g* for 10 min. Both chlorophyll *a* and c_2 were calculated using the equations of Jeffrey and Humphrey (1975) following two 10 h extractions of the crude homogenate in 90% spectroanalyzed acetone in the dark (4°C). No chlorophyll could be detected in the supernatant using this or the more sensitive fluorometric technique of Yentsch and Menzel (1963), indicating that the zooxanthellae had not been disrupted during the homogenization procedure. Superoxide dismutase (SOD) activity of the supernatant, determined by the polarographic technique of Tyler (1975), is expressed in units according to McCord and Fridovich (1969). Catalase activity, expressed in standard Sigma units, was measured using the polarographic procedure of Goldstein (1968). All enzyme assays were performed at 14°C. Soluble protein content of the supernatant was determined using the microbiuret method of Itzhaki and Gill (1964) with bovine serum albumin as the standard.

Enzyme activity in aposymbiotic anemones

Aposymbiotic anemones were collected from several crevices along the jetty and therefore undoubtedly represent multiple clones. Intact anemones were homogenized immediately upon return to the laboratory (No Treatment, Fig. 1A, B) and protein content, SOD, and catalase activities of the supernatant determined as described above. Additional groups of 10 anemones were kept immersed in flowing sea water under the following conditions (Fig. 1A, B): in the dim artificial illumination of the laboratory (maximum irradiance 25 μEinsteins $\cdot$ $m^{-2} \cdot s^{-1}$) (Dim); in dim illumination of the laboratory but continuously exposed to 320 mmHg (0.42 atm) exogenous O_2, a P_{O_2} similar to that measured in tissues of symbiotic anemones in light (Dykens and Shick, 1982) (Dim High O_2); outdoors in full sunlight during the daylight hours (Sun); outdoors in full sunlight but beneath clear sheets of plate glass to block ultraviolet radiation (UV Blocked). After seven days in the above treatments, the anemones were homogenized and chlorophyll extractions performed. Following centrifugation, supernatant protein content and SOD and catalase activities were determined.

Attachment of gravel

Symbiotic anemones from a single high and single low intertidal clone were collected and cleaned of all attached gravel, shell, and other debris using forceps. Groups of 10 anemones were separated immediately into individual aquaria and were kept continuously immersed in running sea water under the following conditions (Fig. 3): outdoors in ambient sunlight (Normal); outdoors in sunlight but beneath plate glass to block ultraviolet wavelengths (UV Blocked); outdoors in sunlight but exposed to 10^{-5} M DCMU (3-3,4-dichlorophenyl)-1,1-dimethylurea), an inhibitor of photosynthesis (DCMU); indoors under the dim illumination of the laboratory (Dim); indoors but continuously exposed to 0.42 atm oxygen (Dim High O_2). *A. elegantissima* preferentially attaches gravel of 1–1.5 mm diameter to the verrucae of its body column (Hart and Crowe, 1977; pers. obs.), and gravel of this size, sieved from the

beach at Bodega Marine Laboratory, was provided to the groups of anemones in a ratio of 5 g gravel to 1 g anemone wet wt. Most (95–99%) of the gravel remained unattached at the end of the experimental period indicating that it had been supplied in excess. After 12 h in the above treatments, the anemones were removed and the attached gravel picked off with forceps, allowed to dry in air for 48 h, and weighed. The anemones were weighed, homogenized, and their chlorophyll contents determined.

Expulsion of zooxanthellae

Anemones from a single mid-intertidal clone from an exposed and brightly sunlit boulder were collected and returned to the laboratory. Fifteen individuals were immediately cleaned of attached debris, homogenized, and their chlorophyll contents determined (No Treatment, Fig. 4). Three other groups of 15 clonemates each were kept continuously immersed outdoors in flowing sea water (Fig. 4). Two of these groups were allowed to retain their natural cover of shell bits and gravel (Debris Attached) and one of these was continuously exposed to 0.42 atm exogenous O_2 (Debris Attached High O_2). The third group was cleaned of all attached debris before being placed outdoors (Debris Removed). After 12 h, anemones from all treatments were cleaned of debris, homogenized, and their chlorophyll contents determined.

Individuals were also collected from a second mid-intertidal clone from a similarly sunny habitat. Chlorophyll content was determined for these field-fresh anemones (No Treatment Clone 2) and after 12 h exposure to 0.42 atm O_2 in the dim illumination of the laboratory (Dim High O_2 Clone 2) (Fig. 4).

Seasonal zooxanthella counts and chlorophyll determinations

An aliquot of the crude homogenate was diluted in an appropriate volume of Moore's calcium-free sea water (Cavanaugh, 1975) plus 0.5 m*M* EDTA. Zooxanthellae were counted using a Neubauer hemocytometer and a Zeiss transmission fluorescence microscope. After digestion in 5% NaOH (12 h, 25°C), total protein content of the homogenate was determined by the dye binding technique of Bradford (1976) using bovine albumin as the standard.

Except for 36 individuals in the month of June (Fig. 5) which were analyzed immediately after collection, all seasonal chlorophyll data are for anemones collected from the jetty and shipped by air to our laboratory in Maine where they were maintained at the habitat temperature in aerated sea water under dim illumination for a maximum of 48 h before homogenization.

Results

Regional enzyme activity

The distribution of chlorophyll among several regions in the anemone supports the subjective impression that most of the endosymbiotic algae are found within the tentacles and oral disc, while fewer are located in the endoderm of the body column and none in the basal disc (Table I). The activities of the protective enzymes SOD and catalase parallel this chlorophyll distribution: the highest SOD and catalase activities are found in the alga-rich tentacles and oral disc, while intermediate levels are found in the body column and the lowest activities in the alga-free basal disc (Table I).

TABLE I

Distribution of total chlorophyll (a + c_2), *superoxide dismutase (SOD) and catalase activities among body regions of the sea anemone* Anthopleura elegantissima

Region	Chlorophyll[1]	SOD[2]	Catalase[2]
Tentacles and oral disc	20.18 ± 2.27	58.87 ± 15.63	150.97 ± 22.13
Body column	1.53 ± 0.61	21.75 ± 7.18	47.07 ± 6.54
Basal disc	0.00	8.73 ± 3.13	34.02 ± 7.42

[1] $\mu g \cdot mg^{-1}$ supernatant protein.
[2] Units $\cdot mg^{-1}$ supernatant protein.
Values given are means ± S.D., n = 5.

Enzyme activities in aposymbiotic anemones

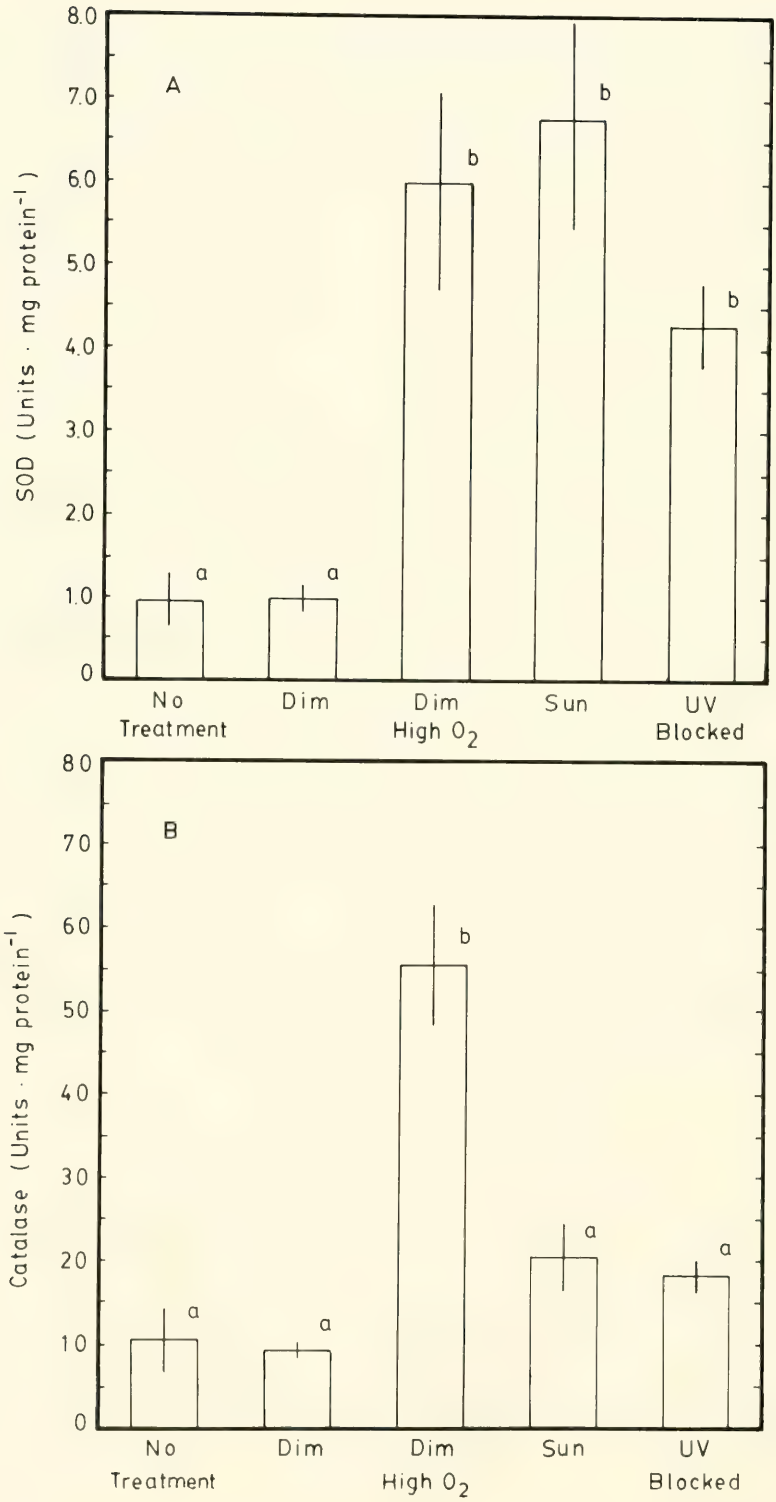
Corroborating previous findings (Dykens and Shick, 1982; Dykens, 1984), SOD activity is low in freshly collected aposymbiotic anemones from shaded habitats (No Treatment, Fig. 1A) and is unchanged after one week maintenance in the dim illumination of the laboratory (Dim, Fig. 1A). However, shade-adapted aposymbiotic anemones exposed to sunlight for one week show a 590% increase in SOD activity ($P < 0.001$) (Sun, Fig. 1A) and a 340% increase ($P < 0.001$) even when protected from UV wavelengths (UV Blocked, Fig. 1A). Similar large increases in SOD activity are induced by exposure to elevated levels of oxygen while in dim illumination (Dim High O_2, Fig. 1A).

Catalase activity of shade-adapted aposymbiotic anemones shows trends similar to SOD activity in that anemones both freshly collected (No Treatment, Fig. 1B) and after one week of laboratory maintenance (Dim, Fig. 1B) have low enzyme activities which can be increased by exposure to sunlight (either with or without UV) or to elevated oxygen. The latter is the most effective (Fig. 1B). No chlorophyll could be detected in any of these anemones after the one week experimental period, indicating that they had not acquired zooxanthellae from other symbiotic anemones in the same flowing sea water system.

It should be noted that the SOD and catalase specific activities reported here are expressed per unit soluble protein in the supernatant and are not directly comparable to our previous data (Dykens and Shick, 1982; Dykens, 1984) where activities are expressed per unit total protein in the anemones.

Attachment of gravel

Continuously immersed high- and low-shore symbiotic anemones, cleaned of debris and placed in sunlight, attach gravel to their body surface in direct proportion to the amount of chlorophyll they contain (Fig. 2 presents data for Normal treatments shown in Fig. 3). Moreover, high-shore anemones (closed circles, Fig. 2; Fig. 3) always attach more gravel per unit chlorophyll than do low-shore anemones (open circles, Fig. 2; Fig. 3) ($F = 65.80$, $n = 100$, $P < 0.0005$) and this is true regardless of experimental treatment (Fig. 3). Although analysis of variance does not reveal significant differences among the four experimental treatment groups of high-shore anemones, all experimental groups of high-shore anemones attach significantly ($P < 0.05$) less gravel than do sunlight-exposed controls (Normal, Fig. 3). In low-shore anemones the extent of gravel attachment can be experimentally manipulated: low-shore anemones in all four treatment groups attach significantly less gravel than


A
SOD (Units · mg protein^{-1})
8.0
7.0
6.0
5.0
4.0
3.0
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a
a
b
b
b
No Treatment
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Dim High O_2
Sun
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B
Catalase (Units · mg protein^{-1})
80
70
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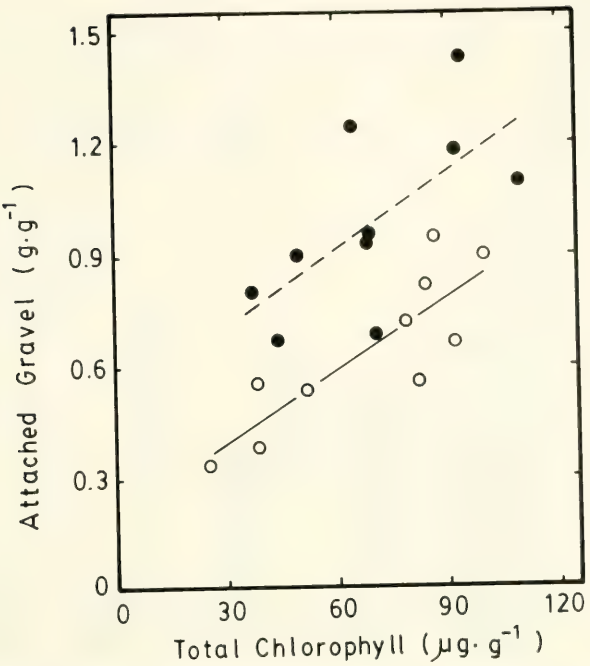

FIGURE 2. Relationship between total chlorophyll ($a + c_2$) content ($\mu g \cdot g^{-1}$ anemone wet wt) and weight of gravel attached to body surface ($g \cdot g^{-1}$ anemone wet wt) by continuously immersed, high-shore (closed circles; $Y = 7.0 \times 10^{-3} X + 0.489$, $r = 0.648$, $P < 0.025$) and low-shore (open circles; $Y = 6.6 \times 10^{-3} X + 0.188$, $r = 0.852$, $P < 0.001$) *A. elegantissima* after 12 h exposure to sunlight.

normal sunlight-exposed controls and this amount is decreased further by inhibiting the photosynthetic production of oxygen either chemically (DCMU) or by lowered irradiance (Dim). Conversely, low-shore anemones in dim illumination, but exposed to elevated exogenous oxygen (Dim High O_2, Fig. 3) attach as much gravel as anemones undergoing photosynthesis in sunlight without UV. Both low- and high-shore anemones exposed to full sunlight, but protected from UV (UV Blocked, Fig. 3), attach significantly ($P < 0.05$) less gravel than clonemates exposed to sunlight containing UV wavelengths (Normal, Fig. 3).

Gravel as sunscreen

The effectiveness of attached debris as a sunscreen is shown by the zooxanthella expulsion data presented in Figure 4. When allowed to retain their attached debris (Debris Attached), anemones from this mid-intertidal clone lose no significant amount of chlorophyll after 12 h of sunlight exposure compared to field-fresh clonemates (No Treatment). During the same time period, however, clonemates in sunlight, but de-

FIGURE 1. A. Superoxide dismutase (SOD), and B. catalase activities in freshly collected, shade-adapted aposymbiotic *A. elegantissima* (No Treatment) and after one week exposure to dim illumination in the laboratory (Dim), full sunlight (Sun), sunlight without UV (UV Blocked), and dim illumination but constant exposure to 320 mmHg O_2 (Dim High O_2). Mean ± S.D., n = 10. Means not significantly different at $P < 0.001$ (one way analysis of variance; Student-Newman-Keuls multiple comparison test) share superscripts.

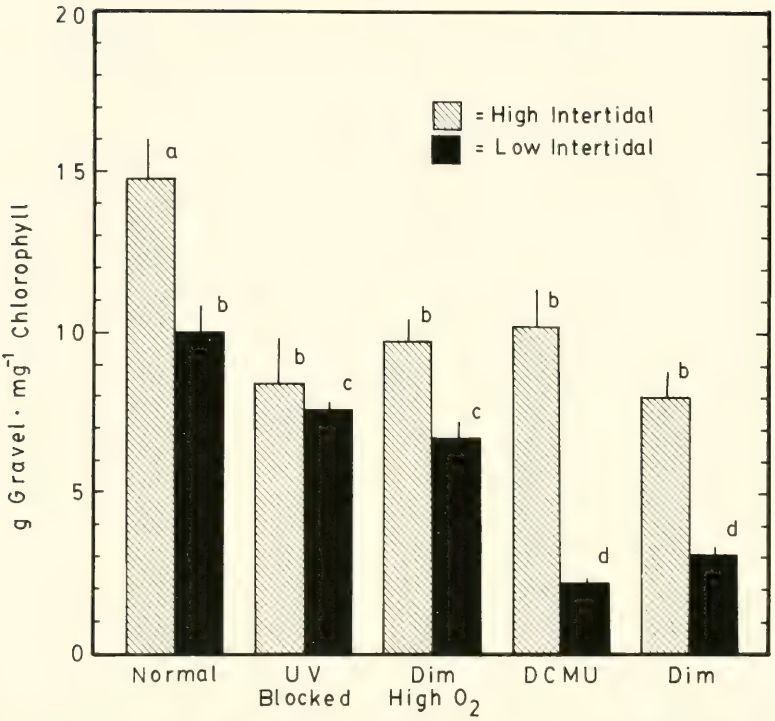

FIGURE 3. Gravel attachment by high-shore (hashmarked) and low-shore (solid bars) symbiotic *A. elegantissima* after 12 h exposure to gravel under various conditions (see text for full explanation of treatments). Mean ± S.D., n = 10. Means not significantly different at $P < 0.001$ (two-way analysis of variance; Student-Newman-Keuls) share superscripts.

prived of attached cover (Debris Removed), lose significant ($P < 0.01$) amounts of chlorophyll. This loss of zooxanthellae also can be induced by continuously exposing sunlit anemones with debris attached to 320 mmHg oxygen (Debris Attached High O_2, Fig. 4). To test whether elevated O_2 alone is the proximal cause of the observed expulsion of zooxanthellae, anemones from a second clone from a habitat similar to that of the first were continuously exposed to 0.42 atm O_2 under dim illumination for 12 h. These anemones (Dim High O_2 Clone 2, Fig. 4) lose no significant amount of chlorophyll compared to freshly collected clonemates (No Treatment Clone 2, Fig. 4).

Seasonal changes in numbers of zooxanthellae and chlorophyll content

All experiments reported here and previously (Shick, 1981; Dykens, 1984; Shick and Dykens, 1984) were conducted in June of 1980, 1982, and 1983, a month chosen to minimize air/sea water temperature differences (Barbour *et al.*, 1973). This also coincides with the period of least difference in chlorophyll content between high- and low-shore anemones (Fig. 5A).

There is a seasonal cycle in anemone chlorophyll content that varies inversely with mean daily solar radiation (Fig. 5A). Seasonal differences are more pronounced in low-shore anemones, but both low- and high-shore individuals contain more total chlorophyll during the winter months, when irradiance is at its lowest, and less chlo-

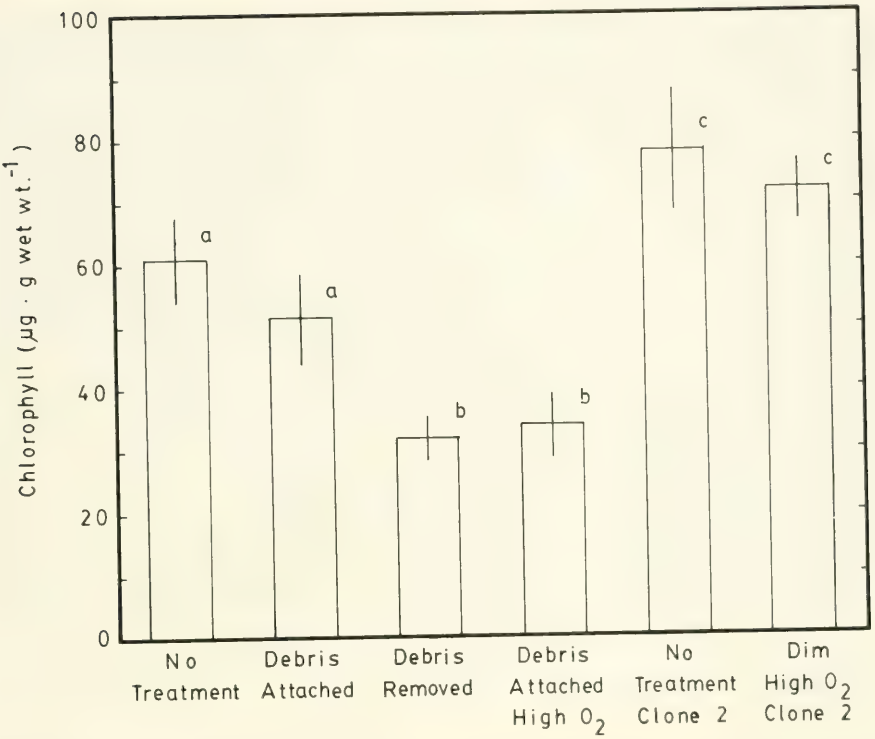

FIGURE 4. Effect of debris attached to the body surface on expulsion of zooxanthellae from *A. elegantissima* after 12 h under various conditions (see text for explanation of treatments). Mean ± S.D., n = 15. Means not significantly different at $P < 0.001$ (one way analysis of variance; Student-Newman-Keuls) share superscripts.

rophyll during the brighter period of June to September (Fig. 5A). Although the total chlorophyll content of low-shore anemones varies about 5-fold between September and December, the number of algal cells in *A. elegantissima* remains fairly constant throughout the year (Fig. 5B). Both chlorophylls *a* and c_2 increase as average daily solar radiation decreases, but chlorophyll c_2 increases disproportionately which reduces the chlorophyll $a{:}c_2$ ratio during the winter months (Fig. 5B).

DISCUSSION

The sea anemone *Anthopleura elegantissima* shows biochemical and behavioral adaptations that allow it to exploit the nutritional benefits provided by its photosynthetic endosymbionts while minimizing the potential of oxygen toxicity and photodynamic damage. As a long-term adaptation to avoid the cytotoxicity of molecular oxygen, this anemone maintains SOD and catalase activity in direct proportion to its chlorophyll content, an estimator of potential oxygen production (Dykens, 1984). Moreover, the localization within the anemone of these enzymes which act to minimize oxygen toxicity by maintaining low cellular levels of O_2^- and H_2O_2 (Fridovich, 1978), directly parallels regional chlorophyll distributions (Table I). Most of the chlorophyll, and consequently most of the oxygen production, is found in the tentacles and oral disc which also contain the highest activities of the two protective enzymes (Table I).

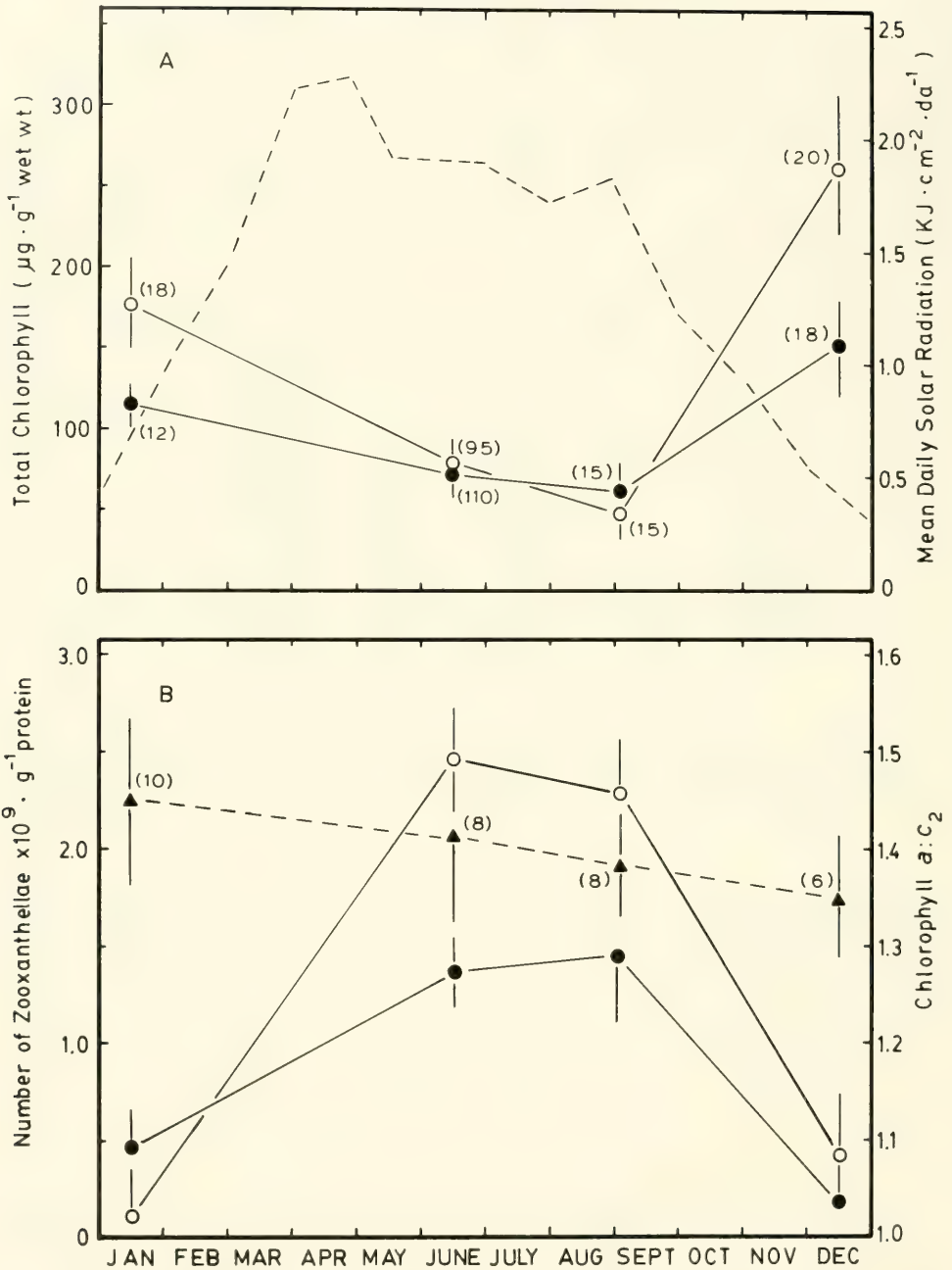

FIGURE 5. A. Seasonal fluctuation in total chlorophyll ($a + c_2$) content ($\mu g \cdot g^{-1}$ anemone wet wt) of high-shore (closed circles) and low-shore (open circles) *A. elegantissima* (Mean ± S.D., n indicated) as well as average daily solar radiation at the collection site (dashed line). Irradiance data redrawn from Barbour *et al.* (1973) with permission. The plateau in mean daily solar radiation seen from mid-May to September is due to prevalent fog in June, July, and August, which is normal for this period (Barbour *et al.*, 1973). B. Seasonal variation in number of zooxanthellae (number algal cells $\cdot g^{-1}$ total protein) isolated from low-shore *A. elegantissima* (solid triangles) (Mean ± S.E., n indicated) and chlorophyll $a{:}c_2$ ratios for high-shore (closed circles) and low-shore (open circles) anemones. (Mean ± S.E., n same as for total chlorophyll in Fig. 5A.)

FIGURE 6. Localized retraction of tentacles and contraction of radial muscles in the oral disc by immersed *Anthopleura xanthogrammica* in response to bright sunlight (550 μEinsteins $\cdot m^{-2} \cdot s^{-1}$). Note that the shaded portion of the anemone remains expanded, as does the entirely shaded anemone at left. Also note the gravel attached to the verrucae of the body column.

In addition to these enzymatic defenses, *A. elegantissima* shows behavioral responses that reduce exposure to intense sunlight. During intervals of high irradiance, this anemone retracts its alga-containing tentacles and contracts its marginal sphincter which constricts the oral disc (Pearse, 1974; Shick and Dykens, 1984). This behavior, which shades the majority of the zooxanthellae thereby reducing the production of oxygen, occurs in response to elevated levels of oxygen and to sunlight exposure *per se* (Shick and Dykens, 1984). It is a finely modulated behavior in that immersed anemones partially exposed to sunlight retract only those tentacles and contract that area of the oral disc that are directly illuminated, behaviors perhaps mediated by local electrical conduction systems (Marks, 1976; McFarlane, 1984) or by the effects of light on the muscles themselves (North and Pantin, 1958), while the shaded portion of the anemone remains expanded (shown in the congeneric *A. xanthogrammica* in Fig. 6).

Reduced photosynthesis ($P_z$net) during periods of peak irradiance, due to contraction in *A. elegantissima,* is also seen in symbiotic corals (Porter, 1980) and tridacnid clams (Mangum and Johansen, 1982) and will reduce estimates of the contribution made by zooxanthellae to animal respiration (CZAR). The impact of reduced $P_z$net on CZAR is only partially offset by concurrent reductions in animal respiration while contracted (Shick and Dykens, 1984). Both of these factors reinforce the need for long-term monitoring of O_2 flux under natural intensities of irradiance if any appraisal of the nutritional status of symbiotic associations is to be environmentally realistic.

Even when *A. elegantissima* is fully contracted, the superficial tissues of the body column remain exposed to direct sunlight and possible photodynamic damage. Moreover, the zooxanthellae within the column are also exposed to sunlight and the sessile anemone has no means, other than attaching a shading sunscreen (see below), to modulate O_2 production by these algae. However, there is five times more SOD

activity and four times more catalase activity in the body column of *A. elegantissima* than predicted on the basis of the enzyme to chlorophyll ratios observed in the tentacles and oral disc. These inordinately high levels of enzyme activities may reflect an additional need for cellular defenses against photogenerated oxygen radicals above those required by the amount of chlorophyll (and subsequent O_2 production) in this region.

Supporting the suggestion that SOD and catalase are defenses against photodynamic damage mediated by oxygen radicals is the finding that aposymbiotic anemones, previously unexposed to direct sunlight, show a 590% increase in SOD activity and a 100% increase in catalase activity following a one week exposure to sunlight (Fig. 1). In fact, sunlight exposure is as effective as exposure to hyperbaric oxygen in inducing SOD activity in aposymbiotic anemones (Fig. 1A). Less energetic wavelengths of visible light are responsible for most of the increase in SOD activity: anemones exposed to sunlight, but protected from UV, possess fully two-thirds the SOD activity of anemones exposed to sunlight containing UV.

Catalase activity shows a qualitatively similar, if somewhat less spectacular, pattern. The trend towards increased catalase activity seen in sun-exposed anemones is also seen in anemones protected from UV (Fig. 1B), again indicating that these enzymic responses are due primarily to visible wavelengths of light. It may be that the catalytic capacity of pre-existing catalase is nearly adequate to cope with the increased production of H_2O_2 due to the sun-induced increase in SOD activity as well as the normal production of H_2O_2 from other cellular processes (Dykens, 1984). This is not the case, however, following chronic exposure to hyperbaric oxygen: anemones indoors but exposed to 0.42 atm O_2 show a 5-fold increase in catalase activity. Perhaps exposure to elevated oxygen increases H_2O_2 production from metabolic processes that are independent of SOD activity such as increases in glucose oxidase activity and oxidations involving reduced flavoproteins.

In addition to the inordinately robust enzymic defences in the column tissues, *A. elegantissima* attaches gravel, calcareous fragments, and pieces of macroalgae to the verrucae of the body column. This attached debris not only effectively decreases desiccation during aerial exposure (Hart and Crowe, 1977), but also serves as a sunscreen that shades the body column and reduces O_2 production by algae in the column as well as the potential for photodynamic damage. A preliminary analysis of 20 anemones from a single clone, 10 from the sunny side and 10 from the shady side of a mid-intertidal boulder, indicated that the more brightly illuminated anemones attached more debris (0.80 mg per mg anemone dry wt $\pm$ 0.16 S.D., *versus* 0.43 mg per mg anemone dry wt $\pm$ 0.20; $t = 4.51$, $P < 0.001$). Since these anemones were routinely exposed to air, it could be argued that the principal role of the light-colored gravel was to reflect sunlight and to maintain a low body temperature (which would also reduce evaporation) in the exposed anemones. However, continuously immersed anemones from both low- and high-shore clones attach gravel in direct proportion to the amount of chlorophyll they contain (Fig. 2). In fact, for low-shore anemones which experience less desiccation, photosynthetic production of oxygen appears to be the proximal determinant of gravel attachment, although exposure to UV also plays a role (Fig. 3). High-shore anemones, however, consistently attach a great deal of gravel regardless of experimental treatment. It may be that in these anemones, which are conditioned to aerial exposure, the requirement for gravel as a defense against desiccation is paramount, so that they attach it whenever it is available. Even these high-shore anemones, however, attach less gravel when protected from UV.

The attached debris is an effective sunscreen. Anemones allowed to retain their cover also retain their zooxanthellae when exposed to full sunlight, while clonemates deprived of debris expel half their algal complement under the same conditions

(Fig. 4). This loss of zooxanthellae appears to involve an interaction between irradiance and high intracellular O_2 levels: sun-exposed anemones with debris attached expel their algae only when exposed to hyperbaric O_2, while anemones exposed to identical oxygen levels, but kept under low illumination, retain their algae.

The seasonal fluctuations in anemone chlorophyll content, which varies inversely with average solar radiation, cannot be attributed to corresponding changes in the number of zooxanthellae, but are due to changes in the chlorophyll content of a fairly constant standing crop of algal cells (Fig. 5A, B). These seasonal fluctuations are more pronounced in low-shore compared to high-shore anemones, perhaps due to the compounding factor of seasonally changing sea water turbidity and corresponding attenuation of sunlight reaching more deeply submerged low-shore clones. Also, high-shore anemones are directly exposed to sunlight at low tide, which may limit the amount of chlorophyll they can safely contain. Regardless, algae from both high- and low-shore anemones contain more chlorophyll during the winter months (Fig. 5A), probably not as a response to lower sea water temperatures which vary annually by only 6°C (Barbour *et al.*, 1973), but as an adaptation which enhances use of the seasonally reduced solar flux. Although both chlorophylls *a* and c_2 increase as average daily irradiance decreases, disproportionate increases in chlorophyll c_2 relative to chlorophyll *a* (Fig. 5B) imply that photoacclimatization of zooxanthellae in *A. elegantissima* occurs primarily *via* proliferation of accessory antenna pigments within existing photosynthetic units (PSUs) (Prézelin, 1981). There are no O_2 flux data available directly comparing maximum rates of photosynthesis of winter and summer acclimatized anemones. If found to exist, seasonally similar levels of maximum O_2 production would support the proposition that algal photoacclimatization is essentially complete and that it occurs *via* increases in the size, not in the number, of PSUs as is the case for zooxanthellae from other (Dustan, 1982; Chang *et al.*, 1983), but not all (Chang *et al.*, 1983), symbiotic associations.

Seasonally altering chlorophyll concentration represents a means whereby the zooxanthellae can maximize photosynthetic potential in a changing photic environment on a long-term basis. However, maintaining chlorophyll levels sufficient to maximize photosynthetic capacity under average light conditions may expose the host anemone during brief periods of peak irradiance to levels of hyperbaric oxygen and photodynamic interactions that are capable of overwhelming enzymic defenses. *A. elegantissima* responds to these short-term increases in photon flux behaviorally, by contracting and attaching an effective sunscreen of debris to its exposed body surface.

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LITERATURE CITED

BARBOUR, M. G., R. B. CRAIG, F. R. DRYSDALE, AND M. T. GHISELIN. 1973. *Coastal Ecology Bodega Head.* U. Calif. Press, Berkeley. 338 pp.

BRADFORD, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**: 248–254.

CADET, J., AND R. TEOULE. 1978. Comparative study of oxidation of nucleic acid components by hydroxyl radicals, singlet oxygen and superoxide anion radicals. *Photochem. Photobiol.* **28:** 661–667.

CAVANAUGH, G. M. 1975. *Formulae and Methods VI of the Marine Biological Laboratory Chemical Room.* Marine Biological Laboratory, Woods Hole. 84 pp.

CHANG, S. S., B. B. PRÉZELIN, AND R. K. TRENCH. 1983. Mechanisms of photoadaptation in three strains of the symbiotic dinoflagellate *Symbiodinium microadriaticum. Mar. Biol.* **76:** 219–229.

CHAPMAN, D. J., AND J. W. SCHOPF. 1983. Biological and biochemical effects of the development of an aerobic environment. Pp. 302–320 in *Earth's Earliest Biosphere,* J. W. Schopf, ed. Princeton Univ. Press, Princeton.

CLAYTON, R. K. 1977. *Light and Living Matter, Volume 2: The Biological Part.* Robert E. Krieger Publ. Co., Huntington, New York. 243 pp.

COOK, C. B. 1983. Metabolic interchange in algae-invertebrate symbiosis. *Int. Rev. Cytol. Suppl.* **14:** 177–210.

COOPER, W. J., AND R. G. ZIKA. 1983. Photochemical formation of hydrogen peroxide in surface and ground waters exposed to sunlight. *Science* **220:** 711–712.

DUSTAN, P. 1982. Depth-dependent photoadaptation by zooxanthellae of the reef coral *Montastrea annularis. Mar. Biol.* **68:** 253–264.

DYKENS, J. A. 1984. Enzymic defenses against oxygen toxicity in marine cnidarians containing endosymbiotic algae. *Mar. Biol. Lett.* **5:** 291–301.

DYKENS, J. A., AND J. M. SHICK. 1982. Oxygen production by endosymbiotic algae controls superoxide dismutase activity in their animal host. *Nature* **297:** 579–580.

DYKENS, J. A., AND J. M. SHICK. 1983. Protection against photosynthetic oxygen by animals containing endosymbiotic algae. *Am. Zool.* **24:** 978.

FEE, J. A. 1980. Is superoxide toxic? Pp. 41–48 in *Biological and Clinical Aspects of Superoxide and Superoxide Dismutase,* W. H. Bannister and J. V. Bannister, eds. Elsevier, Amsterdam.

FRIDOVICH, I. 1977a. Chemical aspects of superoxide radical and of superoxide dismutases. Pp. 3–12 in *Biochemical and Medical Aspects of Active Oxygen,* O. Hayaishi and K. Asada, eds. Univ. Tokyo Press, Tokyo.

FRIDOVICH, I. 1977b. Oxygen is toxic. *Bioscience* **27:** 462–466.

FRIDOVICH, I. 1978. The biology of oxygen radicals. *Science* **201:** 875–880.

VAN GINKEL, G., AND J. K. RAISON. 1980. Light-induced formation of O_2^- oxygen radicals in systems containing chlorophyll. *Photochem. Photobiol.* **32:** 793–798.

GOLDSTEIN, D. B. 1968. A method for assay of catalase with the oxygen electrode. *Anal. Biochem.* **24:** 431–437.

HALLIWELL, B. 1977. Superoxide and hydroxylation reactions. Pp. 335–349 in *Superoxide and Superoxide Dismutases,* A. M. Michelson, J. M. McCord, and I. Fridovich, eds. Academic Press, New York.

HALLIWELL, B. 1978. Biochemical mechanisms accounting for the toxic action of oxygen on living organisms: The key role of superoxide dismutase. *Cell Biol. Int. Rept.* **2:** 113–129.

HALLIWELL, B. 1982. The toxic effects of oxygen on plant tissues. Pp. 89–123 in *Superoxide Dismutase, Vol. 1,* L. W. Oberley, ed. CRC Press Inc., Boca Raton, FL.

HART, C. E., AND J. H. CROWE. 1977. The effect of attached gravel on survival of intertidal anemones. *Trans. Am. Microsc. Soc.* **96:** 28–41.

HILL, H. A. O. 1978. The superoxide ion and the toxicity of molecular oxygen. Pp. 173–208 in *New Trends in Bio-inorganic Chemistry,* R. J. P. Williams and J. R. R. F. DaSilva, eds. Academic Press, New York.

ITZHAKI, R. F., AND D. M. GILL. 1964. A micro-biuret method for estimating proteins. *Anal. Biochem.* **9:** 401–410.

JEFFREY, S. W., AND G. F. HUMPHREY. 1975. New spectrophotometric equations for determining chlorophylls *a, b,* c_1, and c_2 in higher plants, algae and natural photoplankton. *Biochem. Physiol. Pflanz.* **167:** 191–194.

JOKIEL, P. L. 1980. Solar ultraviolet radiation and coral reef epifauna. *Science* **207:** 1069–1071.

JOKIEL, P. L., AND R. H. YORK, JR. 1982. Solar ultraviolet photobiology of the reef coral *Pocillopora damicornis* and symbiotic zooxanthellae. *Bull. Mar. Sci.* **32:** 301–315.

KHAN, A. U. 1978. Activated oxygen: Singlet molecular oxygen and superoxide anion. *Photochem. Photobiol.* **28:** 615–627.

MANGUM, C. P., AND K. JOHANSEN. 1982. The influence of symbiotic dinoflagellates on respiratory processes in the giant clam *Tridacna squamosa. Pacific Sci.* **36:** 395–401.

MARKS, P. S. 1976. Nervous control of light responses in the sea anemone, *Calamactis praelongus. J. Exp. Biol.* **65:** 85–96.

MCCLOSKEY, L. R., D. S. WETHEY, AND J. W. PORTER. 1978. Measurement and interpretation of photosynthesis and respiration in reef corals. *Monogr. Oceanogr. Methodol.* (UNESCO) **5:** 379–396.

MCCORD, J. M., AND I. FRIDOVICH. 1969. Superoxide dismutase—an enzymic function for erythrocuprein (hemocuprein). *Biol. Chem.* **244:** 6049–6055.

McFarlane, I. D. 1984. Nerve nets and conducting systems in sea anemones: two pathways excite tentacle contractions in *Calliactis parasitica*. *J. Exp. Biol.* **108:** 137–149.

Muscatine, L. 1980. Productivity of zooxanthellae. Pp. 381–402 in *Primary Productivity in the Sea,* P. G. Falkowski, ed. Plenum Publ. Corp., New York.

Muscatine, L., L. R. McCloskey, and R. E. Marian. 1981. Estimating the daily contribution of carbon from zooxanthellae to coral animal respiration. *Limnol. Oceanogr.* **26:** 601–611.

Muscatine, L., P. G. Falkowski, and Z. Dubinsky. 1984. Carbon budgets in symbiotic associations. In *International Colloquium on Endocytobiology, Vol. 2,* W. Schwemmler and H. A. E. Schenk, eds. W. de Gruyter, Berlin. (In press.)

North, W. J., and C. F. A. Pantin. 1958. Sensitivity to light in the sea-anemone *Metridium senile* (L.): adaptation and action spectra. *Proc. R. Soc. Lond. B.* **148:** 385–396.

Pearse, V. B. 1974. Modification of sea anemone behavior by symbiotic zooxanthellae: expansion and contraction. *Biol. Bull.* **147:** 641–651.

Porter, J. W. 1980. Primary productivity in the Sea: reef corals *in situ.* Pp. 402–410 in *Primary Productivity in the Sea,* P. G. Falkowski, ed. Plenum Publishing Corp., New York.

Prézelin, B. B. 1981. Light reactions in photosynthesis. *Can. Bull. Fish. Aquat. Sci.* **210:** 1–43.

Shick, J. M. 1981. Heat production and oxygen uptake in intertidal sea anemones from different shore heights during exposure to air. *Mar. Biol. Lett.* **2:** 225–236.

Shick, J. M., and J. A. Dykens. 1984. Photobiology of the symbiotic sea anemone *Anthopleura elegantissima:* Photosynthesis, respiration and behavior under intertidal conditions. *Biol. Bull.* **166:** 608–619.

Steele, R. D. 1976. Light intensity as a factor in the regulation of the density of symbiotic zooxanthellae in *Aiptasia tagetes* (Coelenterata, Anthozoa). *J. Zool. Lond.* **179:** 387–405.

Steinman, H. M. 1982. Superoxide dismutases: Protein chemistry and structure-function relationships. Pp. 11–68 in *Superoxide Dismutase Vol. I,* L. W. Oberley, ed. CRC Press, Boca Raton, FL.

Sullivan, S. G., C. C. Winterbourn, and A. Stern. 1983. Hypothesis: Oxygen, superoxide dismutase and catalase form a metabolic pathway that protects against oxidative damage in the red blood cell. Pp. 364–367 in *Oxy Radicals and their Scavanger Systems. Volume I: Molecular Aspects,* G. Cohen and R. A. Greenwald, eds. Elsevier, Amsterdam.

Trench, R. K. 1979. The cell biology of plant-animal symbiosis. *Ann. Rev. Plant. Physiol.* **30:** 485–531.

Tyler, D. D. 1975. Polarographic assay and intracellular distribution of superoxide dismutase in rat liver. *Biochem. J.* **147:** 493–504.

Yentsch, C. S., and D. W. Menzel. 1963. A method for the determination of phytoplankton chlorophyll and phaeophytin by fluorescence. *Deep-Sea Res.* **10:** 221–231.

Reference: *Biol. Bull.* **167**: 698–703. (December, 1984)

DIGESTION AND DISTRIBUTION OF TRIPALMITOYLGLYCEROL IN *DIPLODON DELODONTUS* (MOLLUSCA, BIVALVIA)

GRACIELA A. HUCA, RICARDO J. POLLERO[1], AND RODOLFO R. BRENNER[2]

Instituto de Investigaciones Bioquímicas de La Plata (INIBIOLP), Facultad de Ciencias Médicas, Universidad Nacional de La Plata, calles 60 y 120, (1900) La Plata, Argentina

Abstract

Fat digestion, absorption, and transport in the fresh water mollusc *Diplodon delodontus* were studied after 1 and 6 h of force-feeding ^{14}C tripalmitoylglycerol and 1-^{14}C palmitic acid. In a 1 h period, the mollusc was able to hydrolyze more than 50% of the triacylglycerol to free acids monoacyl and diacylglycerols. Digestion and absorption was completed before the 6 h feeding, and most of the label was eliminated. Hydrolysis occurred primarily in the stomach. The mollusc absorbed the products of hydrolysis and apparently triacylglycerol molecules, too. During the period of tripalmitoylglycerol digestion (1 h), the labeled palmitic acid was transported by the hemolymph in the free acid, monoacylglycerol, and triacylglycerol fractions that were also the principal components found in the stomach. During the post absorption period (6 h) the label was principally bound to triacylglycerols. When 1-^{14}C palmitic acid was fed to *D. delodontus,* the acid was absorbed and the label transported exclusively in the free acid fraction of the hemolymph during the first hour. At 6 h ~75% was still transported as free acid and the rest as triacylglycerol. The free palmitic acid was incorporated in the soft tissues of the mollusc and slowly esterified to triacylglycerols.

Introduction

Since Yonge's (1926) work, several reports have been published regarding lipid digestion in bivalve molluscs. These are generally based on histological and histochemical methods. The original controversy between intracellular or extracellular digestion of fats apparently ended when George (1952) found evidence employing histochemical techniques that extracellular hydrolysis of triacylglycerol took place in the stomach of lamellibranchia molluscs. Some authors suggested that lipolytic enzymes might be localized in vesicles excreted by digestive glands (Mansour and Zari, 1946; Morton, 1956) while others also detected lipase activity in the crystalline style (Hozumi, 1959; 1961; Payne, 1978; Palmer, 1979). Patton and Quinn (1973) reported a quantitative study on lipid digestion in a marine clam. Using homogenates of the crystalline style of *Spisula solidissima,* they investigated optimal conditions for lipase activity and showed that it catalyzed the hydrolysis of a wide variety of substrates but had low activity for triacylglycerols.

This information indicates that the digestion of lipids is not yet fully understood in bivalve molluscs and that little is known about the absorption and distribution of dietary lipids. To trace lipids, the fresh water mollusc *Diplodon delodontus* was force-

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[1] Member of Carrera del Investigador Científico, Comisión de Investigaciones Científicas de la Provincia de Buenos Aires.

[2] Member of Carrera del Investigador Científico, Consejo Nacional de Investigaciones Científicas y Técnicas, Argentina.

fed ^{14}C tripalmitoylglycerol and 1-^{14}C palmitate. Tripalmitoylglycerol was chosen since Pollero *et al.* (1983) and Irazú *et al.* (1984) have shown that palmitoyl glycerides are the predominant lipids in the natural diet of the mollusc. Free acid was also administered to investigate triacylglycerol resynthesis from the acid absorbed in the digestive tract.

Materials and Methods

Materials

Adult *Diplodon delodontus* (10–12 cm) were collected from a pond close to the La Plata River (34°53′ S, 57°50′ W) Berisso, Buenos Aires Province, Argentina at 1.5 m depth. Specimens were maintained at room temperature in aquaria with aerated water and pond sediment for a week before the experiments were performed.

Tripalmitoylglycerol (^{14}C) (51.5 mCi/mmol) labeled in carbon 1 of palmitic acid and 1-^{14}C palmitic acid (58 mCi/mmol) (Amersham International Ltd., England) were used as radioactive tracers. Non-radioactive tripalmitoylglycerol and palmitic acid (Nu-Check Prep, U.S.A.) were used to dilute labeled materials for the experiments.

Feeding with labeled substrates

V-shaped holes were cut in the shells of the molluscs with a hand saw to expose the mouth. Animals survived this treatment for periods up to 24 h. Each clam was fed, with a water suspension of 6.25 μCi (3.1 μmol) tripalmitoylglycerol and soy bean powder (52 mg), through a canula located in the mouth. Two groups of four animals each were maintained in these conditions for one and six hours, respectively. After that time the shell valves were opened, cutting the aductor muscles with a bistoury, and the hemolymph was immediately obtained by cardiac puncture. Approximately 1 ml of hemolymph was recovered per animal. The stomach and its content, intestine with its content, and digestive diverticula were carefully dissected. All other soft tissues were assembled in one pool. Two other groups of four clams each were also fed with ammonium palmitate (3 μCi/0.5 μmol each clam) for one and six hours. The procedures followed for the substrate administration and dissection of tissues were similar to those described for the tripalmitoylglycerol assays.

Lipid extraction and analysis

Dissected organs and the remaining soft tissues were homogenized in a Tri-R homogenizer with chloroform-methanol (2:1 v/v) at the rate of 20 ml per gram of tissue and total lipids extracted by the procedure of Folch *et al.* (1957). Radioactivity was measured in aliquots of the extracts dissolved in Bray's scintillation solution. Total lipids were analyzed by thin-layer chromatography on plates of Silicagel G plus 10% boric acid, using hexane-diethyl ether-acetic acid (60:40:1.5 v/v) as development solvents. Lipids were identified on the plates by comparing the R_f with the corresponding standards. The radioactivity distribution among the lipids was measured on the plates with a scanner counter apparatus (Berthold, Germany). Peak areas were calculated by triangulation.

Results

Tripalmitoylglycerol

Table I shows the distribution of radioactivity found in different organs of the clam after force-feeding ^{14}C tripalmitoylglycerol for one and six hours. After the first

TABLE I

Radioactivity distribution in Diplodon delodontus *tissues fed* ^{14}C *tripalmitoylglycerol*

Tissues	Total radioactivity (d.p.m. × 10^3)		Lipid radioactivity (d.p.m. × 10^3/mg of lipid)		Radioactivity recovered in the lipids (percent)[1]	
	1 h	6 h	1 h	6 h	1 h	6 h
Stomach	32,333.4	100.5	1530.9	28.7	53.9	0.2
Intestine	4748.7	95.0	949.7	25.0	7.9	0.2
Digestive diverticula	1232.7	2197.0	94.8	22.5	2.1	3.5
Hemolymph	4435.7	72.0	252.0	51.4	7.4	0.1
Other soft tissues	3386.3	3932.0	6.9	8.5	5.6	6.4

Total radioactivity administered per pool was 55,000 × 10^3 d.p.m.
Results were obtained from pools of four animals.
[1] Percent of administered radioactivity.

hour of force-feeding the labeled lipid, approximately half of the radioactivity administered still remained in the stomach. Only ~13% was recovered in tissues not belonging to the digestive tube. After 6 h only 10% of the administered radioactivity was recovered and only a small percentage (0.2%) remained in the stomach. Soft tissue lipids collected the highest amount. Therefore, absorption of the lipid was already complete at 6 h and a large part of the palmitic acid had been consumed or eliminated with the feces.

To investigate the fate of the labeled tripalmitoylglycerol, the radioactivity distribution in the lipids of each tissue separated by thin-layer chromatography was determined (Table II). In the stomach, at the first hour less than 50% of the labeled palmitate of tripalmitoylglycerol appeared in the triglyceride fraction and the rest in the products of hydrolysis, principally free palmitic acid and less proportions in monoacylglycerols and diacylglycerols. These results indicate that lipase digestion of

TABLE II

Percent distribution of radioactivity in the lipids of Diplodon delodontus *after 1 and 6 h feeding* ^{14}C *tripalmitoylglycerol*

Tissue	Period (h)	Free fatty acids	Monoacyl-glycerols	Diacyl-glycerols	Triacyl-glycerols
Stomach	1	40.0	11.4	5.7	42.9
	6	22.6	2.0	—	75.4
Intestine	1	9.3	6.2	6.7	77.8
	6	4.4	4.1	—	91.5
Digestive diverticula	1	7.3	6.1	3.9	82.7
	6	9.0	7.1	—	83.9
Hemolymph	1	44.8	27.2	—	28.0
	6	4.4	5.5	—	90.1
Other soft tissues	1	7.5	—	—	92.5
	6	5.3	8.4	4.2	82.0

No label was found in phospholipids.
Results were obtained from pools of four animals.

the tripalmitoylglycerol occurred in the stomach during the first hour. The same products of hydrolysis were found in the intestine and digestive diverticula but in lesser amounts and percentages.

After 6 h the small amount of radioactivity in the stomach mainly belonged to the triacylglycerol fraction and only a low percentage was recovered in the free acids and monoacylglycerols (Table II). A similar distribution with even less labeling in products of hydrolysis was found in intestine and digestive diverticula.

The lipids of hemolymph and other soft tissues were also labeled in the first hour of the experiment. Except for hemolymph in which most of the label was found in fractions with R_f corresponding to monoacylglycerols and free fatty acids, more than 90% of the radioactivity in the other tissues was recovered in the triacylglycerols. Therefore, in the first hour after food intake, part of the triacylglycerol fed had been digested, the products absorbed, and distributed in the different organs.

After 6 h few radioactive lipids remained in hemolymph (Table I) and these consisted principally of triacylglycerols (Table II). In the other tissues most of the radioactivity was also found in triacylglycerols.

Palmitic acid

The above experiments gave some information about digestion and absorption of a triacylglycerol by the mollusc. However, they did not provide definitive information about the resynthesis of triacylglycerols in the digestive tube tissues from free fatty acids. For this reason, molluscs were force-fed the ammonium salt of 1-^{14}C palmitic acid. Table III shows that after the first hour of administration all radioactivity found in the digestive tube, organs, and hemolymph remained unaltered as free acid. A similar behavior has been reported for longer time periods in *Crassostrea gigas* (Allen and Conley, 1982). However, in other organs a small part of the label was also attached to triacyl and diacylglycerols. Therefore, no esterification of the absorbed acid occurred under these conditions in the digestive tube cells and the acid was transported as such by the hemolymph. Only target tissues would esterify part of the acid to synthesize neutral lipids.

TABLE III

Percent distribution of radioactivity in the lipids of Diplodon delodontus *after 1 and 6 h-feeding 1-^{14}C palmitic acid*

Tissue	Period (h)	Free fatty acids	Monoacyl-glycerol	Diacyl-glycerol	Triacyl-glycerol
Stomach	1	100	—	—	—
	6	74.7	—	7.1	18.2
Intestine	1	98.4	—	—	1.6
	6	83.7	—	2.0	14.3
Digestive diverticula	1	100	—	t	t
	6	61.7	—	9.9	28.4
Hemolymph	1	100	—	—	t
	6	76.5	—	—	23.5
Other soft tissues	1	90.3	t	4.7	5.0
	6	71.9	0.6	7.6	19.9

No label was found in the phospholipids.
Results were obtained from pools of four animals.

After 6 h of 1-^{14}C palmitate ingestion, the incorporation of the label in triacylglycerols and diacylglycerols in the digestive tract organs was detected (Table III). But even at this time most of the palmitic acid remained unesterified. The hemolymph still transported the palmitic acid predominantly in the free form but 23.5% was now detected in the triacylglycerols. In the other organs, most of the label was still detected in the free acid but the fraction esterified to diacyl- and triacylglycerols had increased.

Discussion

After force-feeding ^{14}C tripalmitoylglycerol, the radioactivity was found in diacylglycerol, monoacylglycerol, and free fatty acids of the stomach (Table II). Consequently, the presence of these lipids demonstrates the existence of hydrolytic activity in this part of the digestive tube and confirms earlier experiments done with other molluscs (Hozumi, 1959; Patton and Quinn, 1973; Payne, 1978; Palmer, 1979). Nonenzymatic hydrolysis was eliminated since the incubation of 1-^{14}C tripalmitoylglycerol with deoxycholate and $CaCl_2$ in phosphate buffer pH 7.1 for 1 h at 25°C did not produce labeled free acids. In intestine and digestive diverticula the proportion of hydrolysis products of the triacylglycerol is remarkably lower suggesting that the largest part of the tripalmitoylglycerol lipolysis took place in the stomach. However, this experiment does not eliminate the possibility of lipase activity in intestine and diverticula produced by enzymes arriving from the stomach or even produced in these tissues.

The present data do not establish clearly in which part of the digestive tube dietary lipids are absorbed. However, the high lipase activity of the stomach in addition to the abundant villi and rich vascularization in the stomach wall (Huca *et al.*, 1982) suggest that most lipid absorption takes place at this level.

Tables I to III indicate that absorbed lipids are distributed by the hemolymph to the different organs. Tables II and III indicate that hemolymph reflects approximately the lipid composition of the stomach. After the first hour of feeding, the tripalmitoylglycerol, triacylglycerol, diacylglycerol, monoacylglycerol, and free fatty acids of the gastric tube were labeled except for the diacylglycerols; the same lipids in a rather similar proportion were found in the hemolymph.

When the free fatty acid was administered (Table III) the radioactivity was detected only in the free acid fraction of the hemolymph. Therefore, these data indicate that the digestive tube walls absorb free palmitic acid (Tables II, III) and monopalmitoylglycerol (Table II), and they can also be transported without transformation by the hemolymph. Therefore, the mechanism of fatty acid transport in mollusc hemolymph would differ significantly from mammals that carry ingested fatty acids in triacylglycerol molecules incorporated to chylomicrons. They would even differ from more related animals, *e.g.*, insects that carry them as diacylglycerols in specific lipoproteins (Fichera and Brenner, 1982).

However, labeled triacylglycerols were found in *D. delodontus* hemolymph after 1-^{14}C tripalmitoylglycerol was administered in the food (Table II). This triacylglycerol present in the hemolymph could be of two origins. It could be produced by resynthesis in the mucous cells of the digestive tube wall from the labeled products derived from the hydrolysis of administered ^{14}C tripalmitoylglycerol. This reaction takes place in mammals in which monoacylglycerol and diacylglycerols may be converted to triacylglycerols by acylation in the intestine wall. Absorbed free acids are also converted to acyl-CoA and then esterified to glycerol phosphate produced in glycolysis or monoacylglycerols and transformed at the end into triacylglycerols (Johnston, 1963). Alternatively, one should consider the direct absorption of tripalmitoylglycerol. When 1-^{14}C palmitic acid was administered to the mollusc for 1 h,

the label was found in the free acid and not in the triacylglycerols of the hemolymph (Table III), suggesting little esterification of tagged free palmitic acid to triacylglycerols in the digestive tube cells. In view of these results, the presence of labeled triacylglycerols in the hemolymph of the mollusc after 1 h of force-feeding 1-^{14}C tripalmitoylglycerol (Table II) would suggest the second alternative which considers that part of triacylglycerol molecules could be directly absorbed through the digestive walls by the *Diplodon delodontus.*

However, in the 6-h experiment, administered 1-^{14}C palmitic acid also labeled a triacylglycerol fraction of hemolymph, stomach, intestine, and diverticula. Therefore, the incorporation of the free acid into triacylglycerols would be produced at longer periods, but we can not yet establish which could be the responsible tissue for the esterification.

The increase of time in the experiment also increased the labeling of triacylglycerols of other soft tissues indicating very probably a local biosynthesis from the free acid provided by the hemolymph, since labeled diacylglycerols were also found in these tissues but not in hemolymph (Table III). This triacylglycerol biosynthesis is produced slowly since after 6 h about 62% or more of the label still remained as free palmitic acid.

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LITERATURE CITED

ALLEN, W. V., AND H. CONLEY. 1982. Transport of lipids in the blood of the pacific oyster *Crassostrea gigas* (Thumberg). *Comp. Biochem. Physiol.* **71:** 201–207.

FICHERA, L. E., AND R. R. BRENNER. 1982. Isolation and characterization of the haemolymph lipoproteins of *Triatoma infestans. Comp. Biochem. Physiol.* **72:** 71–75.

FOLCH, J., N. LEES, AND C. H. SLOANE-STANLEY. 1957. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **226:** 497–509.

GEORGE, W. C. 1952. The digestion and absorption of fat in lamellibranchs. *Biol. Bull.* **102:** 118–127.

HOZUMI, M. 1959. On the lipolitic enzyme in the crystalline style of the clam *Venerupis philippinarum* Adams et Reeve. *Sci. Rep. Tokyo Kyoiku Daig.* (Sec. B) **9:** 37–55.

HOZUMI, M. 1961. Studies on the lipase in the digestive diverticula of the clam *Venerupis philippinarum* Adams et Reeve. *Sci. Rep. Tokyo Kyoiku Daig.* (Sec. B) **10:** 155–186.

HUCA, G. A., R. R. BRENNER, AND M. H. NIVEIRO. 1982. A study on the biology of *Diplodon delodontus* (Lamarck, 1819) (Bivalvia hyriidae) I- Ecological aspects and anatomy of the digestive tract. *Veliger* **25:** 51–58.

IRAZÚ, C. E., R. J. POLLERO, AND R. R. BRENNER. 1984. Occurrence of a 22:2 NMI fatty acid and its seasonal distribution among lipids and tissues of the fresh water bivalve *Diplodon delodontus* from an isolated environment. *Lipids.* (In press.)

JOHNSTON, J. 1963. Recent developments in the mechanism of fat absorption. Pp. 105–131 in *Advances in Lipid Research,* Vol. 1, R. Paoletti and D. Kritchevsky, eds. Academic Press, New York.

MANSOUR, D., AND F. G. ZARI. 1946. The digestive diverticula of unioprasidens as organs of secretion. *Proc. Egypt Acad. Sci.* **2:** 38–44.

MORTON, J. E. 1956. The tidal rhythm and action of the digestive system of the lamellibranch *Lasaea rubra. J. Mar. Biol. Assoc. U. K.* **35:** 563–586.

PALMER, R. E. 1979. A histological and histochemical study of digestion in the bivalve *Arctica islandica L. Biol. Bull.* **156:** 115–129.

PAYNE, D. W. 1978. Lipid digestion and storage in the littoral bivalve *Scrobicularia plana* (Da Costa) *J. Molluscan Stud.* **44:** 295–304.

PATTON, J. S., AND J. G. QUINN. 1973. Studies on the digestive lipase of the surf clam *Spisula solidissima. Mar. Biol.* **21:** 59–69.

POLLERO, R. J., C. E. IRAZÚ, AND R. R. BRENNER. 1983. Effect of sexual stages on lipids and fatty acids of *Diplodon delodontus. Comp. Biochem. Physiol.* **76:** 927–931.

YONGE, C. M. 1926. The digestive diverticula in the lamellibranchs. *Trans. Soc. Edin.* **54**(3) No 15.

(3)
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RELATIONSHIPS BETWEEN FREE CUPRIC ION CONCENTRATIONS IN SEA WATER AND COPPER METABOLISM AND GROWTH IN CRAB LARVAE

BRENDA M. SANDERS[1,2] AND KENNETH D. JENKINS[1,3]

[1]*Molecular Ecology Institute, California State University, Long Beach, California, 90840;* [2]*Duke University Marine Laboratory, Beaufort, North Carolina, 28516; and* [3]*Department of Biology, California State University, Long Beach, California, 90840*

Abstract

Crab larvae (*Rhithropanopeus harrisii*) were exposed to a range of free cupric ion concentrations, [Cu^{2+}], regulated in sea water by a metal chelate buffer system. We found a biphasic relationship between intracellular copper distribution and [Cu^{2+}] in sea water. At [Cu^{2+}] within the ambient range ($10^{-12.4}$ to $10^{-10.6}$ *M*), cytosolic copper was associated with both metallothionein (MT) and high molecular weight (HMW) ligands, and was independent of external [Cu^{2+}]. At higher [Cu^{2+}], copper was also associated with very low molecular weight (VLMW) ligands, and accumulated in this ligand pool and the MT pool as external [Cu^{2+}] increased. In marked contrast, copper in the HMW ligand pool did not correlate with [Cu^{2+}] in sea water over the entire range of exposures. Reductions in larval growth occurred at greater than estimated ambient [Cu^{2+}] and correlated with copper accumulation in the MT and VLMW pools.

Introduction

The concentrations and subcellular distributions of metals can provide valuable information on an organism's capacity to adapt to accumulated metals. As a consequence, it has been suggested that the more subtle aspects of metal toxicity in aquatic organisms can be more accurately estimated by examining the distribution of metals among the various intracellular ligand pools (Bayne *et al.*, 1980). However, to understand the ecological significance of these data we must also be able to relate this information on metal metabolism to effects on the organism, the population, and the community (Sanders *et al.*, 1983).

In a previous study on larvae of the crab *Rhithropanopeus harrisii* (Sanders *et al.*, 1983) we used metal-chelate buffer systems to control Cu speciation since the biological availability of Cu is related to the concentration of the free cupric ion, [Cu^{2+}], rather than to the total or chelated Cu concentration (Sunda and Guillard, 1976; Anderson and Morel, 1978; Jackson and Morgan, 1978; Zamuda and Sunda, 1982). These buffers enabled us to expose larvae throughout zoeal development to a range of calculated [Cu^{2+}] while the concentrations of potentially competitive metals were kept constant. In that study we found that most cytosolic Cu was associated with metallothionein, a cysteine-rich metal-binding protein whose synthesis is induced by metals (Hildebrand *et al.*, 1979). This ubiquitous protein has been implicated in metal uptake, metabolism, and detoxication in vertebrates and invertebrates (Richards and Cousins, 1976; Brown *et al.*, 1977; Li *et al.*, 1980; Roesijadi, 1980; Jenkins *et al.*, 1982). We found that the concentration of Cu-thionein in crab larvae was related

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to $[Cu^{2+}]$ in sea water, and an increase in Cu-thionein accumulation correlated with inhibition of larval growth.

In this study we have followed changes in high (HMW) and very low molecular weight (VLMW) Cu binding ligands as well as Cu-thionein. Also, we examined these ligand pools over a wider range of $[Cu^{2+}]$. We report relationships between $[Cu^{2+}]$, Cu-thionein, and larval growth which are similar to our previous findings. In addition, we found that the Cu concentration within the VLMW pool could also be related to the $[Cu^{2+}]$ in sea water, and that increases of Cu in this pool correlated with inhibition of larval growth. In contrast to a current hypothesis (Brown, 1977), we found no correlation between Cu associated with HMW ligands and either external $[Cu^{2+}]$ or inhibition of larval growth. Finally, we found that increases of Cu in the MT and VLMW pools, and inhibition of larval growth, occurred at $[Cu^{2+}]$ just beyond the estimated ambient range in the estuary where the crabs were collected.

Materials and Methods

Gravid females of the mud crab *Rhithropanopeus harrisii* were collected in the Newport River estuary near Beaufort, North Carolina. They were kept in tanks of running sea water and transferred to culture bowls when their eggs were ready to hatch. Newly hatched larvae were exposed to a range of $[Cu^{2+}]$ with metal-nitrotriacetic acid (NTA) buffer systems in sea water (Sunda *et al.*, 1978). The larvae were cultured in the laboratory until they molted to the megalopa stage as described by Costlow *et al.* (1966). Buffered sea water was changed daily. There were 10 larvae per culture bowl and five replicates for each treatment (Sanders *et al.*, 1983).

The metal-NTA buffer system was made up in 35 parts per thousand sea water which had previously been diluted with distilled water to 20 parts per thousand and contained 10^{-4} *M* NTA, 4.6×10^{-8} *M* $ZnCl_2$, 2.4×10^{-8} *M* $CoCl_2$, 1.0×10^{-8} *M* $MnCl_2$, 1.0×10^{-7} $FeCl_3$, and 1.0×10^{-9} *M* Na_2MoO_4. NaOH was added at 4×10^{-4} *M* to adjust the pH to 8.0 ± 0.1. Copper was added as Cu-NTA to achieve concentrations of 1.8×10^{-8} *M* to 5.7×10^{-4} *M*. Free ion concentrations for Cu^{2+} were computed from metal ion-NTA equilibria (Sunda *et al.*, 1978). These concentrations were based on added metal and ranged from $10^{-12.4}$ *M* to $10^{-7.9}$ *M*.

After they molted to the megalopa stage the larvae were sampled, rinsed with distilled water, and lyophilized immediately. Survival and length of time to megalopa were determined as described by Costlow *et al.*, 1966. The freeze-dried megalopa were weighed to the nearest 0.1 μg (Sanders *et al.*, 1984).

Replicate samples were pooled, rehydrated, and homogenized in 0.02 *M* tris-HCl (pH 7.4) with an acid-washed Teflon pestle tissue grinder. The homogenate was centrifuged at $100{,}000 \times g$, and the resulting supernatant was filtered by centrifugation through a 0.2 μm nylon filter. An aliquot (100 μl) of this filtered cytosol was chromatographed on a Toyo Soda steric exclusion (SEC) HPLC column (TSK SW 3000; Sanders *et al.*, 1983). Copper concentrations in the eluant fractions were determined by graphite furnace atomic absorption spectrophotometry (Sanders *et al.*, 1983). Chromatographic data were analyzed by correlation analysis, linear and orthogonal polynomial regression analysis, and analysis of variance (Sokal and Rohlf, 1981). Our confidence limits were set at 0.05.

Results

Cytosolic Cu was associated with three major pools of ligands (Fig. 1): (1) high molecular weight ligands (HMW; ≥20,000 daltons), the majority of which elute with or just behind the void volume; (2) a metallothionein peak (MT) which has an

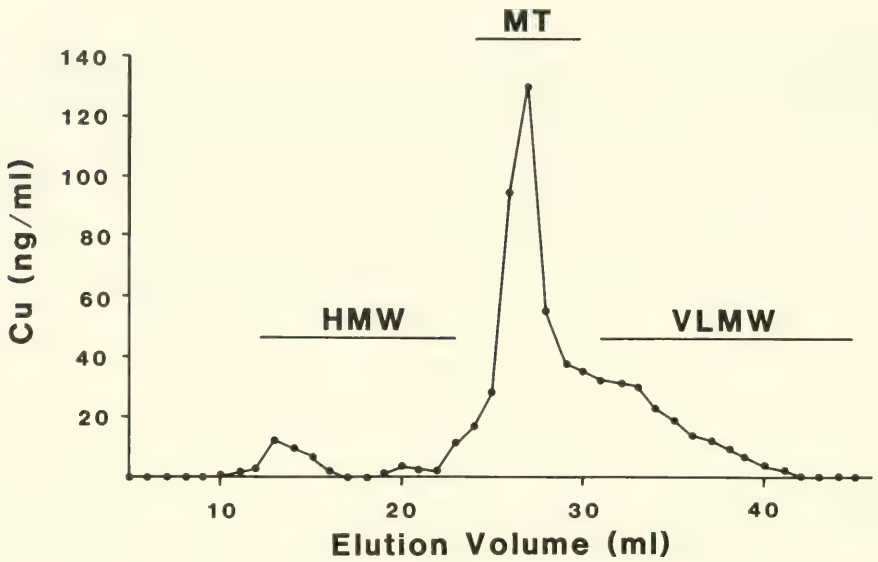

FIGURE 1. Cytosolic distribution of copper in larvae of the crab *Rhithropanopeus harrisii* exposed to free cupric ion concentrations in sea water. HMW, MT, and VLMW represent high molecular weight, metallothionein, and very low molecular weight pools, respectively. The bars represent fractions which were combined for each pool.

apparent molecular weight of 10,000 daltons, and (3) very low molecular weight ligands (VLMW; <5000 daltons) which included molecules too small to be resolved by the column.

There was a biphasic relationship between $[Cu^{2+}]$ in sea water and Cu-thionein and LMW Cu. At $[Cu^{2+}]$ from $10^{-12.68}$ to $10^{-10.6}$ *M* copper metabolism appeared independent of external $[Cu^{2+}]$ (Fig. 2a). At these concentrations there was no significant correlation between external $[Cu^{2+}]$ and Cu-thionein. Copper associated with the VLMW pool was not detectable at environmental concentrations below $10^{-11.0}$ *M*. At concentrations greater than $10^{-10.6}$ *M*, however, Cu in the metallothionein and the VLMW pools correlated significantly with increases in $[Cu^{2+}]$ in sea water ($F = 220$; d.f. = 1, 2; $F = 365$; d.f. = 1, 2). This copper accumulation was reflected in total cytosolic Cu ($F = 363$; d.f. = 1, 2).

The relationship between Cu associated with the HMW ligands and $[Cu^{2+}]$ in sea water contrasted with those of the other pools (Fig. 2b). Although Cu associated with these ligands was significantly above background ($F = 1096$; d.f. = 8, 36), it did not correlate with $[Cu^{2+}]$ in sea water and no accumulation phase was observed (Fig. 2b).

We examined the relationships between $[Cu^{2+}]$ in sea water, survival, duration of the zoeal stages, and megalopa weight to determine the physiological affect of changes in intracellular Cu distribution. Free cupric ion concentrations did not correlate with survival or the duration of zoeal development at concentrations less than $10^{-8.9}$ *M*. At the two highest concentrations tested ($10^{-7.9}$ *M* and $10^{-8.4}$ *M*), all larvae died within three days of exposure. Megalopa weight did correlate with $[Cu^{2+}]$ with significant linear ($F = 53$; d.f. = 1, 36) and quadratic components (Fig. 2c; $F = 50$; d.f. = 1, 36). Megalopa weight was greatest for larvae exposed to $10^{-11.0}$ *M* $[Cu^{2+}]$ and decreased rapidly at higher concentrations.

Copper in the MT and VLMW ligand pools correlated with megalopa weight (Fig. 3; $r^2 = 0.676$; $r^2 = 0.826$). Megalopa weight increased with increases in Cu-

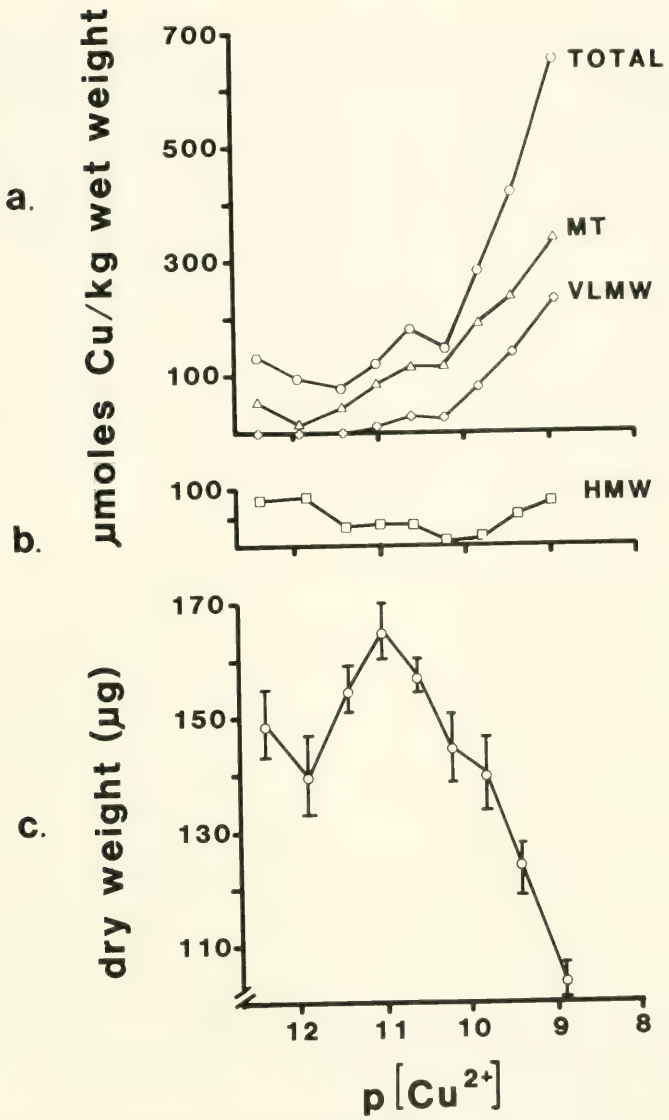

FIGURE 2. (a) Cytosolic distribution of copper expressed in micromoles per kilogram (wet weight) of tissue in *R. harrisii* megalopa exposed to a range of values of $p[Cu^{2+}]$ ($-\log$ of free cupric ion concentration) throughout the duration of larval development. Total, MT, and LMW represent total cytosolic copper, metallothionein, and very low molecular weight ligand pools, respectively. (b) As described above for high molecular weight (HMW) ligand pool. (c) Dry weights of megalopa described above. The vertical lines are one standard error of the mean for five replicates.

thionein until a concentration of 72.6 μm Cu kg^{-1} wet weight ($10^{-11.0}$ *M* exposure), and decreased rapidly at higher concentrations. Although Cu was undetectable in the VLMW pool at the three lowest $[Cu^{2+}]$, it accumulated at higher concentrations. Megalopa weight decreased rapidly with increases of Cu in this pool. In contrast to the MT and VLMW ligand pools, Cu in the HMW pool did not correlate with weight over the entire range of exposures.

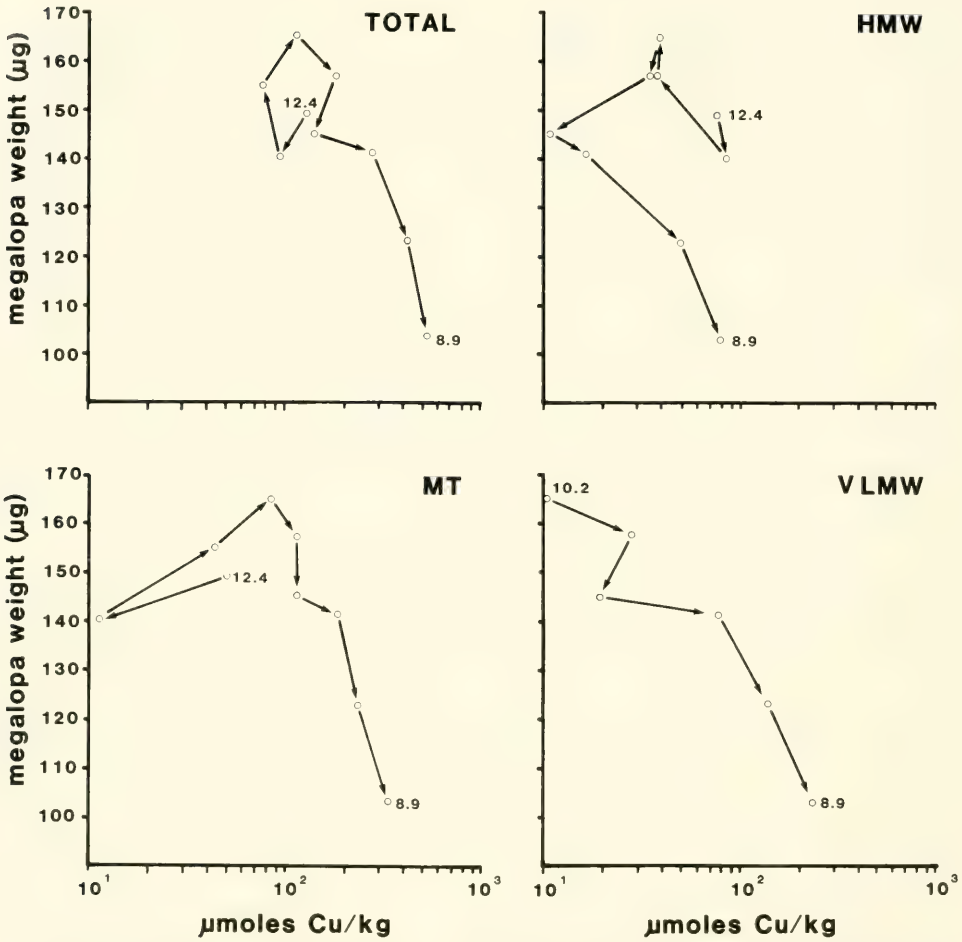

FIGURE 3. Vector diagrams of megalopa dry weight and copper associated with the total cytosol and each cytosolic ligand pool (expressed as micromoles per kilogram dry weight of tissue) in *R. harrisii* megalopa. Total, HMW, MT, and VLMW represent total cytosolic copper, high molecular weight, metallothionein, and very low molecular weight ligands pools, respectively. The arrows delineate the p[Cu^{2+}] (−log free cupric ion concentration) to which the larvae were exposed throughout development from the lowest, to the highest exposures (p[Cu^{2+}] from 12.4 to 8.9).

DISCUSSION

Cytosolic copper accumulation and distribution

The cytosolic distributions of Cu among the HMW, MT, and VLMW ligand pools were similar to those previously reported for *R. harrisii* (Fig. 1; Sanders *et al.*, 1983) and for other crab species (Olafson *et al.*, 1979b; Overnell, 1982). These three metal binding pools have also been reported in other marine invertebrates (Coombs, 1974; Howard and Nickless, 1977, 1978; Jenkins *et al.*, 1982; Frazier and George, 1983).

In our fractionation procedure the HMW pool contained a heterogeneous group of Cu ligands. Most of these ligands are soluble proteins. A number of these proteins, including Cu metalloenzymes and the respiratory pigment hemocyanin, have specific

Cu requirements (Brouwer *et al.*, 1983). Nonspecific binding of Cu to macromolecules in this pool can also occur.

The chemical characterization of metallothioneins in crabs includes amino acid analysis in two species (Olafson *et al.*, 1979a; Overnell, 1982) and amino acid sequencing for one species (Lerch *et al.*, 1981, 1982). Characterization of the MT pool of *R. harrisii* was described in our previous study (Sanders *et al.*, 1983). As with other crab (Olafson *et al.*, 1979a; Overnell and Trewhella, 1979) and mammalian (Kagi and Nordberg, 1979) metallothioneins, it resolves as two major metal binding ligands when fractionated by ion exchange chromatography.

The chemical identity and origin of the VLMW ligand pool is not known but it appears to be made up of a heterogeneous group of Cu complexes. Amino acids may represent an important component of this pool, but these data have yet to be confirmed (Coombs, 1974; Howard and Nickless, 1977, 1978). Frazier and George (1983) have suggested that *in vivo* these VLMW ligands may be associated with granules which become solubilized during homogenization and extraction. Granules containing high concentrations of Cu are present in the hepatopancreas of another crustacean (Weiser and Klima, 1969) but it is not clear if this is the source of the VLMW ligands in crab larvae.

Copper regulation and metabolism

The relationship between $[Cu^{2+}]$ in sea water and cytosolic Cu fell into two discrete patterns (Fig. 2). This biphasic response can be related to the ambient $[Cu^{2+}]$ ($\leq 10^{-10.4}$ *M*) estimated by Sunda and Gillespie (1979) for estuarine waters where the crabs were collected. The first phase occurred at a range of $[Cu^{2+}]$ which is equal to or less than the estimated ambient concentration ($10^{-12.4}$ *M* to $10^{-10.6}$ *M*). In this range of 1.8 orders of magnitude, Cu in the HMW, MT, and VLMW ligand pools were independent of $[Cu^{2+}]$. These data support our previous observations (Sanders *et al.*, 1983) and indicate that Cu accumulation and distribution among the three ligand pools is precisely regulated in crab larvae at exposures at or below ambient $[Cu^{2+}]$.

When larvae are exposed to $[Cu^{2+}]$ which are greater than the ambient concentration ($10^{-10.2}$ *M* to $10^{-8.9}$ *M*), a second phase, one of accumulation, was observed (Fig. 2a). In this phase total cytosolic Cu increased with increases in $[Cu^{2+}]$ in sea water. This accumulation was the result of increases in both Cu-thionein and Cu in the VLMW ligand pool. Accumulation of total cytosolic Cu and Cu-thionein in *R. harrisii* larvae exposed to greater than ambient $[Cu^{2+}]$ has been reported previously (Sanders *et al.*, 1983). In that study cytosolic Cu accumulation and Cu-thionein concentration approached apparent saturation at $[Cu^{2+}]$ just below those which resulted in larval death. This saturation, however, was based on a minimum of data points. In contrast, the data presented here showed no evidence of saturation and the concentrations of total Cu and Cu-thionein attained prior to larval mortality were several fold higher than in the previous experiment (Fig. 3a).

In marked contrast to the other ligand pools, Cu in the HMW ligand pool did not correlate with $[Cu^{2+}]$ in sea water (Fig. 2b). The lack of Cu accumulation in this pool suggests that crab larvae can regulate subcellular Cu distribution over the 3.5 orders of magnitude range of $[Cu^{2+}]$ used in this experiment. Also, the simultaneous increase of Cu in the MT and VLMW ligand pools suggests that they may function as Cu reservoirs which limit nonspecific binding to the HMW ligands.

Free cupric ion concentration and larval growth

In order to understand the ecological significance of Cu accumulation and cytosolic distribution, we examined three parameters: survival, duration of zoeal development,

and megalopa weight. However, only changes in megalopa weight correlated with $[Cu^{2+}]$ (Fig. 2c). Maximum megalopa weight occurred within the range of the estimated ambient $[Cu^{2+}]$ and decreased rapidly at concentrations beyond this range. This pattern of a hormetic overshoot in weight followed by a rapid decrease is an inherent characteristic of stress etiology which has been reported previously in *R. harrisii* larvae (Laughlin *et al.*, 1981; Sanders *et al.*, 1983; 1984). These growth data support our previous observation that fluctuations in ambient $[Cu^{2+}]$ in sea water could limit crab larval growth (Sanders *et al.*, 1983). Cupric ion concentrations near the ambient range also limit growth and nutrient uptake in phytoplankton and bacteria (Jackson and Morgan, 1978; Fitzwater *et al.*, 1982; Sunda and Ferguson, 1983; Jenkins *et al.*, 1984). Thus, natural variations in $[Cu^{2+}]$ could regulate productivity in a wide range of estuarine and marine species through the inhibition of important metabolic processes. The effects of this inhibition could, in turn, modify population dynamics and community composition (Sunda *et al.*, 1981).

Copper accumulation and larval growth

It has been suggested that as long as metals can be sequestered in the MT pool the organism is protected against the toxicity of these metals (Brown *et al.*, 1977, 1983; Jenkins *et al.*, 1982; Nolan and Duke, 1983). However, our data indicate that when larvae were exposed to $[Cu^{2+}]$ beyond the ambient range, the accumulation of Cu in the MT pool was correlated with growth inhibition (Fig. 3).

Although the nonspecific binding of metals to HMW ligands is proposed to be a major mechanism of metal toxicity (Winge *et al.*, 1973; Brown, 1977; Pruell and Engelhardt, 1980), our data show no correlation between Cu in the HMW ligand pool and toxic effects on growth (Fig. 3). Several explanations may account for this apparent discrepency: (1) the techniques used in this study can only detect changes in total Cu and, therefore, do not necessarily distinguish changes in the level of nonspecific binding; (2) the mechanism of metal toxicity may involve the nonspecific binding of Cu to membrane-bound metalloenzymes or macromolecules in other subcellular compartments; and (3) finally Cu uptake and accumulation may modify the uptake and metabolism of other essential metals (*e.g.*, Zn) which may in turn affect larval growth (Bremner and Campbell, 1980; Sunda *et al.*, 1981; Cousins, 1983).

Of the three cytosolic ligand pools, it was the accumulation of Cu in the VLMW pool which was most indicative of toxic effects on the organism. The detection of Cu in this pool was concomitant with perturbations of larval growth. As the Cu concentration in the VLMW pool increased, larval growth rapidly decreased. Cadmium accumulation has been reported in the VLMW pool of the oyster (*Ostrea edulis*) during chronic Cd exposure (Frazier and George, 1983). Although the authors suggest that sequestering cadmium in this pool may protect the oyster from Cd toxicity, they provide no information on the relationship between this accumulation and sublethal effects at the organismic level.

These data emphasize the importance of understanding the interactions among all cytosolic ligand pools. They also underscore the need to correlate these interactions with sensitive indicators of stress (such as growth) before the toxic effects of metals on an organism can be estimated from data on subcellular metal distributions. Finally, these data indicate that changes in intracellular metal metabolism and growth in crab larvae occur at the upper range of the ambient $[Cu^{2+}]$ in the estuary, suggesting that $[Cu^{2+}]$ in sea water may limit larval growth.

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LITERATURE CITED

ANDERSON, D. M., AND F. M. MOREL. 1978. Copper sensitivity of *Gonyaulax tamarensis*. *Limnol. Oceanogr.* **23:** 283–295.

BAYNE, B. L., D. A. BROWN, F. L. HARRISON, AND P. P. YEVICH. 1980. Mussel Health. Pp. 163–235 in *The International Mussel Watch, Report on a Workshop at Barcelona, Spain, December 1978*, E. Goldberg, ed. National Academy of Science, Washington, DC.

BREMNER, I., AND J. K. CAMPBELL. 1980. The influence of dietary copper intake on the toxicity of cadmium. *NYAS* **355:** 319–332.

BROUWER, M., C. BONAVENTURA, AND J. BONAVENTURA. 1983. Metal ion interactions with *Limulus polyphemus* and *Callinectes sapidus* hemocyanins: Stoichiometry and structural and functional consequences of calcium (II), cadmium (II), zinc (II), and mercury (II) binding. *Biochemistry* **22:** 4713–4723.

BROWN, D. A. 1977. Increases of Cd and the Cd:Zn ratio in the high molecular weight protein pool from apparently normal liver of tumor-bearing flounders (*Parophrys vetulus*). *Mar. Biol.* **44:** 203–209.

BROWN, D. A., C. A. BAWDEN, K. W. CHATEL, AND T. R. PARSONS. 1977. The wildlife community of Iona Island jetty, Vancouver, B.C., and heavy-metal pollution effects. *Environ. Conserv.* **4:** 213–216.

BROWN, D. A., R. W. GOSSETT, P. HERSHELMAN, H. A. SCHAEFER, K. D. JENKINS, AND E. M. PERKINS. 1983. Bioaccumulation and detoxification of contaminants in marine organisms from southern California coastal waters. Pp. 171–190 in *Waste Disposal in the Oceans*, D. F. Soule and D. Walsh, eds. Westview Press, Boulder, Colorado.

COOMBS, T. L. 1974. The nature of zinc and copper complexes in the oyster. *Ostrea edulis*. *Mar. Biol.* **28:** 1–10.

COSTLOW, J. D., AND C. G. BOOKOUT. 1971. The effect of cyclic temperatures on larval development in the mudcrab *Rhithropanopeus harrisii*. Pp. 211–220 in *Fourth European Marine Biology Symposium*, D. J. Crisp, ed. Cambridge University Press, London.

COSTLOW, J. D., C. G. BOOKOUT, AND R. J. MONROE. 1966. Studies on the larval development of the crab *Rhithropanopeus harrisii* (Gould). I. The effects of salinity and temperature on larval development. *Physiol. Zool.* **39:** 81–100.

COUSINS, R. J. 1983. Metallothionein—aspects related to copper and zinc metabolism. *J. Inherited Metab. Dis. 6 Suppl.* **1:** 15–21.

FITZWATER, S. E., G. A. KNAUER, AND J. H. MARTIN. 1982. Metal contamination and its effect on primary production measurements. *Limnol. Oceanogr.* **27:** 544–551.

FRAZIER, J. M., AND S. G. GEORGE. 1983. Cadmium kinetics in oysters—a comparative study of *Crassostrea gigas* and *Ostrea edulis*. *Mar. Biol.* **4:** 55–62.

HILDEBRAND, C. E., R. A. TOBEY, E. W. CAMPBELL, AND M. D. ENGER. 1979. A cadmium-resistant variant of the Chinese hamster (CHO) cell with increased metallothionein induction capacity. *Exp. Cell Res.* **124:** 237–246.

HOWARD, A. G., AND G. NICKLESS. 1977. Heavy metal complexation in polluted molluscs. II. Oyster (*Ostrea edulis and Crassostrea gigas*) *Chem. Biol. Interact.* **17:** 257–263.

HOWARD, A. G., AND G. NICKLESS. 1978. Heavy metal complexation in polluted molluscs. III. Periwinkles (*Littorina littorea*), cockles (*Cardium edule*) and scallops (*Chlamys opercularis*). *Chem. Biol. Interact.* **23:** 227–231.

JACKSON, G. A., AND J. J. MORGAN. 1978. Trace metal-chelator interactions and phytoplankton growth in seawater media: Theoretical analysis and comparison with reported observations. *Limnol. Oceanogr.* **23:** 268–282.

JENKINS, K. D., D. A. BROWN, G. P. HERSHELMAN, AND W. C. MEYER. 1982. Contaminant in white croakers *Genyonemus lineatus* (Ayers 1855) from the southern California bight: I Trace metal detoxification/toxification. Pp. 177–196 in *Physiological Mechanisms of Marine Pollutant Toxicity*. W. B. Vernberg, A. Calabrese, F. P. Thurberg, F. J. Vernberg, eds. Academic Press, New York.

JENKINS, K. D., D. A. BROWN, P. S. OSHIDA, AND E. M. PERKINS. 1982. Cytosolic metal distribution as an indicator of toxicity in sea urchins from the southern California bight. *Mar. Pollut. Bull.* **13:** 413–421.

JENKINS, K. D., B. M. SANDERS, AND W. G. SUNDA. 1984. Metal regulation and toxicity in aquatic organisms. Pp. 277–288 in *Mechanisms of Drug and Toxin Action,* T. Singer, T. Mansour, R. Ondarza, eds. Academic Press, New York.

KAGI, J. H. R., AND M. NORDBERG. 1979. *Metallothionein.* Birkhauser Verlag, Basel, Boston, Stuttgart. 378 pp.

LAUGHLIN, R. B., JR., J. NG, AND H. E. GUARD. 1981. Hormesis: A response to low environmental concentrations of petroleum hydrocarbons. *Science* **211:** 705–707.

LERCH, K., D. AMMER, AND R. W. OLAFSON. 1981. Amino acid sequence of crab metallothionein. *FEBS Lett.* **126:** 165–168.

LERCH, K., D. AMMER, AND R. W. OLAFSON. 1982. Crab metallothionein, primary structures of metallothioneins 1 and 2. *J. Biol. Chem.* **257:** 2420–2423.

LI, T. Y., A. J. KRAKER, C. F. SHAW III, AND D. H. PETERING. 1980. Ligand substitution reactions of metallothioneins with EDTA and apo-carbonic anhydrase. *Proc. Natl. Acad. Sci.* **77:** 6334–6338.

NOLAN, C. V., AND E. J. DUKE. 1983. Cadmium accumulation and toxicity in *Mytilus edulis:* involvement of metallothioneins and heavy-molecular weight protein. *Aguat. Toxicol.* **4:** 153–163.

OLAFSON, R. W., R. G. SIM, AND K. G. BOTO. 1979a. Isolation and chemical characterization of the heavy metal-binding protein metallothionein from marine invertebrates. *Comp. Biochem. Physiol.* **62B:** 407–416.

OLAFSON, R. W., A. KEARNS, AND R. G. SIM. 1979b. Heavy metal induction of metallothionein synthesis in the hepatopancreas of the crab *Scylla serrata. Comp. Biochem. Physiol.* **62B:** 417–423.

OVERNELL, J. 1982. Copper metabolism in crabs and metallothionein: *in vivo* effects of copper II on soluble hepatopancreas metal binding components in the crab *Cancer pagurus* containing varying amounts of cadmium. *Comp. Biochem. Physiol.* **73B:** 555–564.

OVERNELL, J., AND E. TREWHELLA. 1979. Evidence for the natural occurrance of (cadmium, copper) metallothionein in the crab *Cancer pagurus. Comp. Biochem. Physiol.* **64C:** 69–76.

PRUELL, R. J., AND F. E. ENGELHARDT. 1980. Liver cadmium uptake, catalase inhibition and cadmium thionein production in the killifish (*Fundulus heteroclitus*) induced by experimental cadmium exposure. *Mar. Envir. Res.* **3:** 101–111.

RICHARDS, M. P., AND R. J. COUSINS. 1976. Influence of parenteral zinc and actinomysin D on tissue zinc uptake and the synthesis of a zinc-binding protein. *Bioinorg. Chem.* **4:** 215–224.

ROESIJADI, G. 1980. Influence of copper on the clam *Protothaca staminea:* effects on gills and occurrence of copper-binding proteins. *Biol. Bull.* **158:** 233–247.

SANDERS, B. M., K. D. JENKINS, W. G. SUNDA, AND J. D. COSTLOW. 1983. Free cupric ion activity in seawater: effects on metallothionein and growth in crab larvae. *Science* **222:** 53–55.

SANDERS, B. M., R. B. LAUGHLIN, JR., AND J. D. COSTLOW. 1984. Growth regulation in larvae of the mud crab, *Rhithropanopeus harrisii.* In *Crustacean Issues, Vol. 2: Crustacean Growth,* A. M. Wenner, ed. A. A. Balkema, Rotterdam. (In press.)

SOKAL, R. R., AND F. J. ROHLF. 1981. *Biometry.* W. H. Freeman and Company, San Francisco. 859 pp.

SUNDA, W. G., AND R. L. FERGUSON. 1983. Sensitivity of natural bacterial communities to additions of copper and to cupric ion activity: a bioassay of copper complexation in seawater. P. 871 in *Trace Metals in Sea Water.* C. Wong, E. Boyle, K. W. Bruland, J. D. Burton, and E. D. Goldberg, eds. Plenum Publishing Corp., New York.

SUNDA, W. G., AND P. A. GILLESPIE. 1979. The response of a marine bacterium to cupric ion and its use to estimate cupric ion activity in seawater. *J. Mar. Res.* **37:** 761–777.

SUNDA, W. G., AND R. R. GUILLARD. 1976. The relationship between cupric ion activity and the toxicity of copper to phytoplankton. *J. Mar. Res.* **34:** 511–529.

SUNDA, W. G., R. T. BARBER, AND S. A. HUNTSMAN. 1981. Phytoplankton growth in nutrient rich seawater: importance of copper-manganese cellular interactions. *J. Mar. Res.* **39:** 567–586.

SUNDA, W. G., D. W. ENGEL, AND R. M. THUOTTE. 1978. Effect of chemical speciation on toxicity of cadmium to grass shrimp, *Palaemonetes pugio:* Importance of free cadmium ion. *Environ. Sci. Technol.* **12:** 409–413.

WEISER, W., AND J. KLIMA. 1969. Compartmentalization of copper in the hepatopancreas of isopods. *Mikroskopie* **24:** 1–9.

WINGE, D., J. KRASNO, AND A. V. COLUCCI. 1973. Cadmium accumulation in rat liver: correlation between bound metal and pathology. Pp. 500–501 in *Trace Element Metabolism in Animals,* Vol. 2, W. G. Hoetkstra, J. W. Suttie, H. E. Ganther, and W. Mertz, eds. University Press, Baltimore.

ZAMUDA, C. D., AND W. G. SUNDA. 1982. Bioavailability of dissolved copper to the American oyster *Crassostrea virginica.* I. Importance of chemical speciation. *Mar. Biol.* **66:** 77–82.

Reference: *Biol. Bull.* **167**: 713–721. (December, 1984)

THE DIMORPHIC CLAWS OF THE HERMIT CRAB, *PAGURUS POLLICARIS*: PROPERTIES OF THE CLOSER MUSCLE

PHILIP J. STEPHENS[1], LISA M. LOFTON[2], AND PETER KLAINER[2]

Villanova University, Department of Biology, Villanova, Pennsylvania 19085

ABSTRACT

The first pair of chelipeds of the flat clawed hermit crab (*Pagurus pollicaris*) are dimorphic. The crusher claw is always on the right side and is larger than the cutter claw on the left. The closer muscle in the crusher claw has a wet weight that is 3.4 times greater than that in the smaller cutter claw.

The closer muscle fiber types are different in the two claws. The crusher closer muscle has fibers with long (9–14 μm) sarcomeres, a high thin to thick filament ratio, and low myofibrillar ATPase activity; these fibers are presumably slow. The cutter closer, by contrast, has two types of fibers that are regionally distributed within the muscle. Fibers located on the dorsal and ventral margins of the muscle have long (8–13 μm) sarcomeres, a high thin to thick filament ratio, low myofibrillar ATPase activity, and are presumably slow fibers. In the central portion of the cutter closer muscle, however, there is a band of fibers with short sarcomeres and high myofibrillar ATPase activity. These features suggest that these fibers are fast. However, the high thin to thick filament ratio found in these "fast" muscle fibers would argue against this presumption. Finally, sarcomere length measurements taken from closer muscle fibers in other thoracic appendages revealed a bimodal distribution of fiber types similar to that observed in the cutter claw.

INTRODUCTION

Although bilateral symmetry is one feature that has been conserved during the evolution of higher animals, some species exhibit bilateral asymmetry. Perhaps the most notable examples can be found in certain crustaceans, where the first pair of chelipeds have become enlarged to form claws. Claw asymmetry can be found in crustaceans such as sexually mature male fiddler crabs (*Uca*), pistol or snapping shrimp (*Alpheus*), the American lobster (*Homarus*), and certain hermit crabs (*Pagurus*). Recently, many investigators have used these crustaceans to study the development of asymmetry in otherwise bilaterally symmetrical animals.

In lobsters and snapping shrimp collected from the wild, as many animals have the major claw on the left side as on the right (Przibram, 1931). Moreover, in these animals the dimorphic claws have different behavioral functions and associated contrasting contractile properties in the claw musculature. In lobsters, for example, the larger "crusher" claw is used for crushing mollusc shells (Herrick, 1895) and has a closer muscle composed of slow tonic fibers capable of producing large amounts of tension (Govind and Lang, 1974; Lang *et al.*, 1977a). The smaller "cutter" claw, by contrast, can be closed rapidly and has a closer muscle with a large proportion of fast, phasic fibers.

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[1] To whom reprint requests should be sent.

[2] Present address: Haverford College, Department of Biology, Haverford, Pennsylvania 19041.

In snapping shrimp, the smaller "pincer" claw is used for manipulation of small objects, while the larger "snapper" claw is used during territorial encounters with other shrimp (Schein, 1975). The pincer claw closer muscle is composed of intermediate and fast muscle fibers (Stephens and Mellon, 1979), which are regionally distributed within the muscle (O'Connor *et al.*, 1982); the fast fibers form a central band along the longitudinal axis of the muscle. The snapper claw closer muscle, however, is composed of a uniform population of slow, tonic fibers. An interesting feature of adult snapping shrimp is an ability to reverse claw configuration (Wilson, 1903; Mellon and Stephens, 1978). Removal or denervation of the snapper claw causes the contralateral pincer claw to enlarge and transform into a snapper claw. The process involves modifications of the external skeletal arrangements and an increase in the mass of the claw musculature. In addition, in Floridian species, there is a change in the properties of the closer muscle fibers from fast and intermediate to slow (Mellon and Stephens, 1980), and there is a parallel increase in the degree of facilitation exhibited by the excitatory motor neurons (Stephens and Mellon, 1979). These results have provoked the suggestion that neurotrophic influences underlie claw transformation in snapping shrimp.

In the dimorphic claws of hermit crabs (*Pagurus pollicaris*) the larger claw is always found on the right side. This may be explained by the right-handed spiral of the gastropod shells in which they live. Such a shell orientation would exert less spatial constraint on the development of the claw on the right side. In the present investigation we have examined the fiber composition of the closer muscles in the dimorphic claws using morphological, histochemical, and biochemical properties and find them to be different.

Materials and Methods

Flat clawed hermit crabs (*Pagurus pollicaris*) were obtained commercially from Woods Hole, Massachusetts, or from Panacea, Florida. In the laboratory, the animals were kept in aquaria filled with artificial sea water at a temperature of 15–20°C. The animals were fed frozen brine shrimp or Tetramin fish food, and were usually used for experimentation within two weeks of arrival at the laboratory. The study involved analysis of muscle fibers types by examining myofibrillar adenosinetriphosphatase (ATPase) activity from frozen sections (Ogonowski and Lang, 1979; O'Connor *et al.*, 1982), and by measuring sarcomere length from teased myofibrils (Atwood, 1973, 1976; Josephson, 1975).

The histochemical properties of the closer muscles in pairs of claws were examined using standard techniques (Ogonowski and Lang, 1979; O'Connor *et al.*, 1982). Since the cuticle of the propus is thick, it was necessary to remove the shell surrounding the closer muscle prior to freezing in liquid nitrogen and sectioning.

Sarcomere length measurements were made for the closer muscles in all of the thoracic appendages, using methods described elsewhere (Lang *et al.*, 1977a; O'Connor *et al.*, 1982). Briefly, the animal was removed from its shell and made to autotomize a limb by applying pressure at the base. The limb was soaked in a saline solution (Chapple, 1977) in which the calcium ions had been replaced with magnesium, to prevent measurement errors due to muscle contraction. The dactyl was fixed in the open position and the limb was bathed in alcoholic Bouin's solution for two days. The limb was then stored in 80% ethanol. Individual fibers were removed from the closer muscle and were teased apart in 80% ethanol on a microscope slide. The myofibrils were examined under a compound microscope fitted with Nomarski optics. A calibrated ocular micrometer was used to measure the length of five consecutive

sarcomeres; at least three measurements were made for each muscle fiber and the average length of a sarcomere was calculated for each muscle fiber.

Electron microscopy was performed on the closer muscle of the cutter and crusher claws. The cuticle on the ventral surface of the propus together with the underlying opener muscle were removed from an autotomized claw. Small holes were drilled through the cuticle of the propus and the dactyl was fixed in the open position. The claw was soaked in the calcium-free saline and then fixed for 60 min in a solution containing glutaraldehyde (2.5%), formaldehyde (0.2%), sucrose (300 m*M*), sodium chloride (60 m*M*), calcium chloride (2 m*M*), and sodium cacodylate (150 m*M*, pH 7.2). Individual muscle fibers were removed from the closer muscle and fixed in the same solution for an additional 30–60 min. The muscle fibers were washed in the same solution (without fixatives), post-fixed in 2% osmium tetroxide, and embedded in epon for thin sectioning on an ultramicrotome.

Results

The crusher claw on the right side is larger than the contralateral cutter claw (Fig. 1A, B) and has a closer muscle of greater mass. In seven pairs of claws the wet weight of the closer muscle in the crusher claw was 3.4 (S.D. ± 0.3) times larger than in the cutter claw (cutter closer muscle weight 27.5–141 mg).

Measurement of sarcomere lengths of fibers in the crusher closer muscle gave values between 9 and 14 μm (Fig. 1F). The homogeneous population of long sarcomeres indicates that the closer muscle in the larger crusher claw is composed of slow fibers (Atwood, 1973, 1976; Josephson, 1975). In the cutter closer muscle, by contrast, sarcomere length analysis revealed a bimodal distribution of fiber types: short sarcomere (2–4 μm), presumably fast fibers, and long sarcomere (8–13 μm), presumably slow fibers. Moreover, data from fibers selectively removed from different regions of the cutter closer muscle showed that fast fibers are located primarily in the central portion of the muscle (Fig. 1E), although some are found ventrally (Fig. 1C). Slow fibers, however, are situated on the dorsal and ventral margins of the muscle (Fig. 1C, D).

Table I gives closer muscle sarcomere length data for the five pairs of thoracic appendages from one animal; for each muscle, fibers were removed from dorsal, ventral, and central regions. It is interesting that in the walking (2 and 3) and the vestigial (4 and 5) limbs there is a bimodal distribution of fiber types, which is similar to that observed in the cutter claw closer muscle.

Electron microscopy of closer muscle fibers from the cutter and crusher claws confirmed the sarcomere length data obtained from light microscopy. In addition, cross sections permitted analysis of the relationship between the thick and thin filaments. In long sarcomere fibers removed from cutter and crusher claw closer muscles each thick filament is associated with 10–14 thin filaments (Fig. 2A–D). This arrangement is typical of slow or tonic muscle (Atwood, 1973). In cutter closer muscle fibers with short sarcomeres (Fig. 2E), presumably fast fibers, each thick filament is associated with 7–10 thin filaments (Fig. 2F). This arrangement of thick and thin filaments is not typical of fast phasic fibers (Atwood, 1973).

Figure 3 shows frozen transverse sections of a closer muscle from a cutter claw stained for myofibrillar ATPase activity. Fibers located in the central region of the cutter closer muscle were dark staining, while those on the dorsal margin were generally light staining. On the ventral margin of the muscle some fibers stained darkly while others were light staining for myofibrillar ATPase activity. It has been shown in other crustacean muscles that fibers with high myofibrillar ATPase activity (*i.e.*, dark staining) are phasic or fast, while light staining fibers are tonic or slow (Ogonowski and Lang,

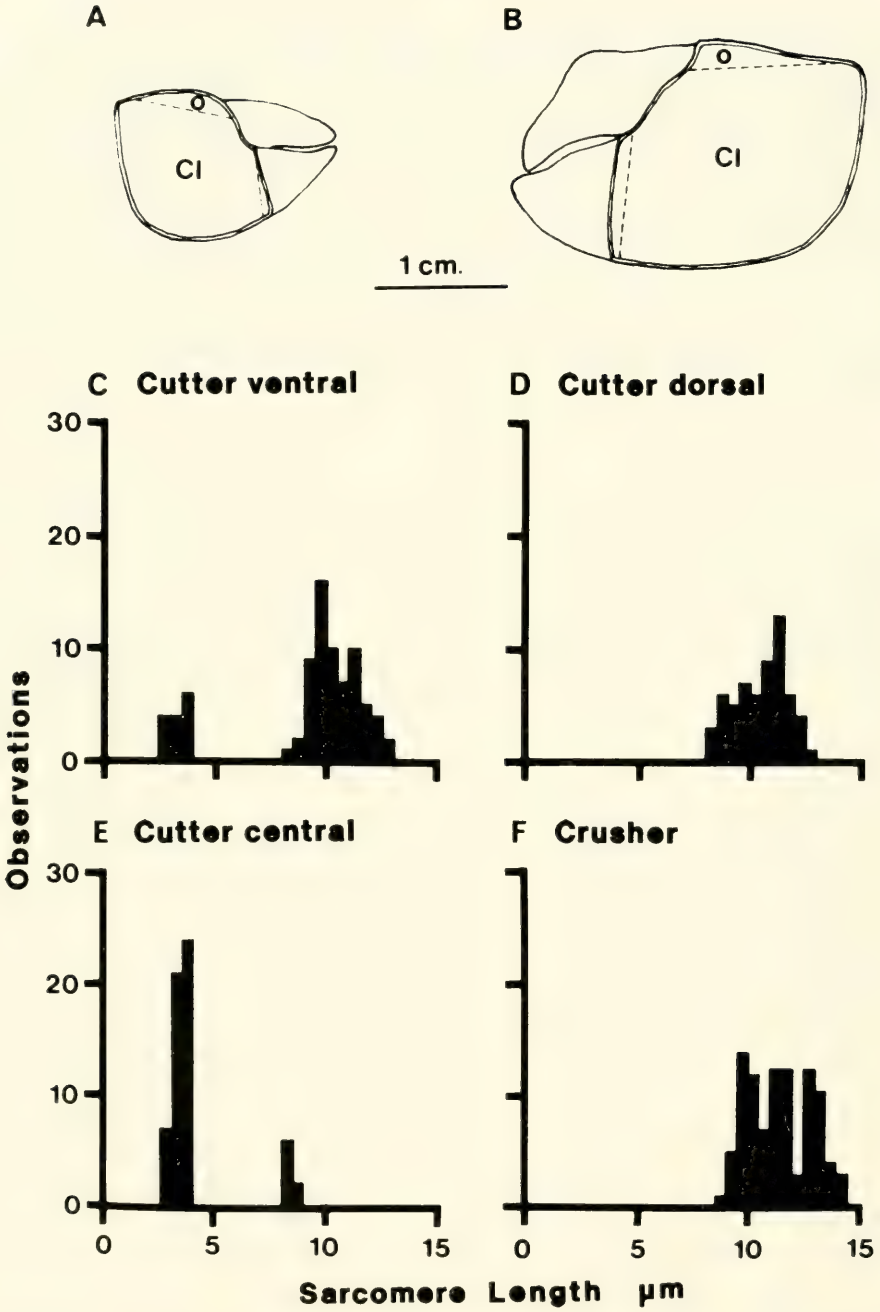

FIGURE 1. Sarcomere length measurements for closer muscle fibers in the crusher and cutter claws of a hermit crab. A, B: Diagrams of the cutter and crusher claws, respectively, showing their relative sizes and the locations of the closer (Cl) and opener (O) muscles. C–F: The sarcomere lengths of closer muscle fibers removed from different regions of the clutter claw (judged by eye) and from the crusher claw. Measurements were made from 250 fibers in the cutter and 100 fibers in the crusher claw.

TABLE I

The sarcomere lengths of closer muscle fibers from the five thoracic limbs of a hermit crab (Pagurus pollicaris)[1]

Thoracic limb	Right	Left
1	11.6 (1.7)	10.3 (1.3)
		3.6 (0.5)
2	10.1 (1.3)	10.0 (0.5)
3	3.8 (0.4)	4.4 (0.3)
	9.5 (0.4)	9.4 (0.3)
4	3.8 (0.1)	4.2 (0.3)
	9.6 (0.2)	9.4 (1.1)
5	4.2 (0.3)	4.3 (0.2)
	8.6 (0.4)	8.4 (0.4)
	4.2 (0.2)	4.3 (0.2)

[1] The sarcomere lengths of 25 fibers in each muscle were measured and the values represent mean sarcomere lengths (± standard deviation). Note that the first thoracic limb on the right side is a crusher and on the left is a cutter.

1979; Silverman and Charlton, 1980; O'Connor *et al.*, 1982). Our results, coupled with the sarcomere length measurements (Figs. 1, 2), indicate that the crusher closer muscle is composed of slow tonic fibers. The cutter closer has slow fibers on the dorsal and ventral margins, and fast fibers located ventrally and centrally, forming a band along the longitudinal axis of the muscle. Our data, therefore, demonstrate a regional distribution of different fiber types in the cutter claw closer muscle of the hermit crab. However, the distribution is not as discrete as that found in the closer muscles of the smaller claws of snapping shrimp (O'Connor *et al.*, 1982) and lobster (Ogonowski *et al.*, 1980), where the fast fibers form discrete bands in the muscle.

DISCUSSION

The present study of the dimorphic claws of the hermit crab (*Pagurus pollicaris*) shows that the closer muscles have different morphological and histochemical characteristics, from which certain contractile properties can be concluded (Atwood, 1973). The crusher closer muscle is composed of an homogeneous population of slowly contracting tonic fibers, while the cutter closer has two types of fibers, fast and slow (Figs. 1–3). The different types of fibers are regionally distributed within the cutter closer so that the fast fibers form a band along the longitudinal axis of the claw, in the central and ventral portions of the muscle (Fig. 3). However, it is interesting that the presumptive fast cutter closer muscle fibers, although they have high myofibrillar ATPase activity and short sarcomeres, also have a thick to thin filament ratio that is not typical of fast fibers. To our knowledge this is the first report of a fast adult crustacean that contrasts with the "classical" arrangement of one thick filament surrounded by six thin filaments fibers (Atwood, 1973).

It is interesting that a similar regional distribution of fiber types has been found in the closer muscle of the smaller chelipeds of other crustaceans with dimorphic claws, most notably snapping shrimp (O'Connor *et al.*, 1982) and lobsters (Ogonowski

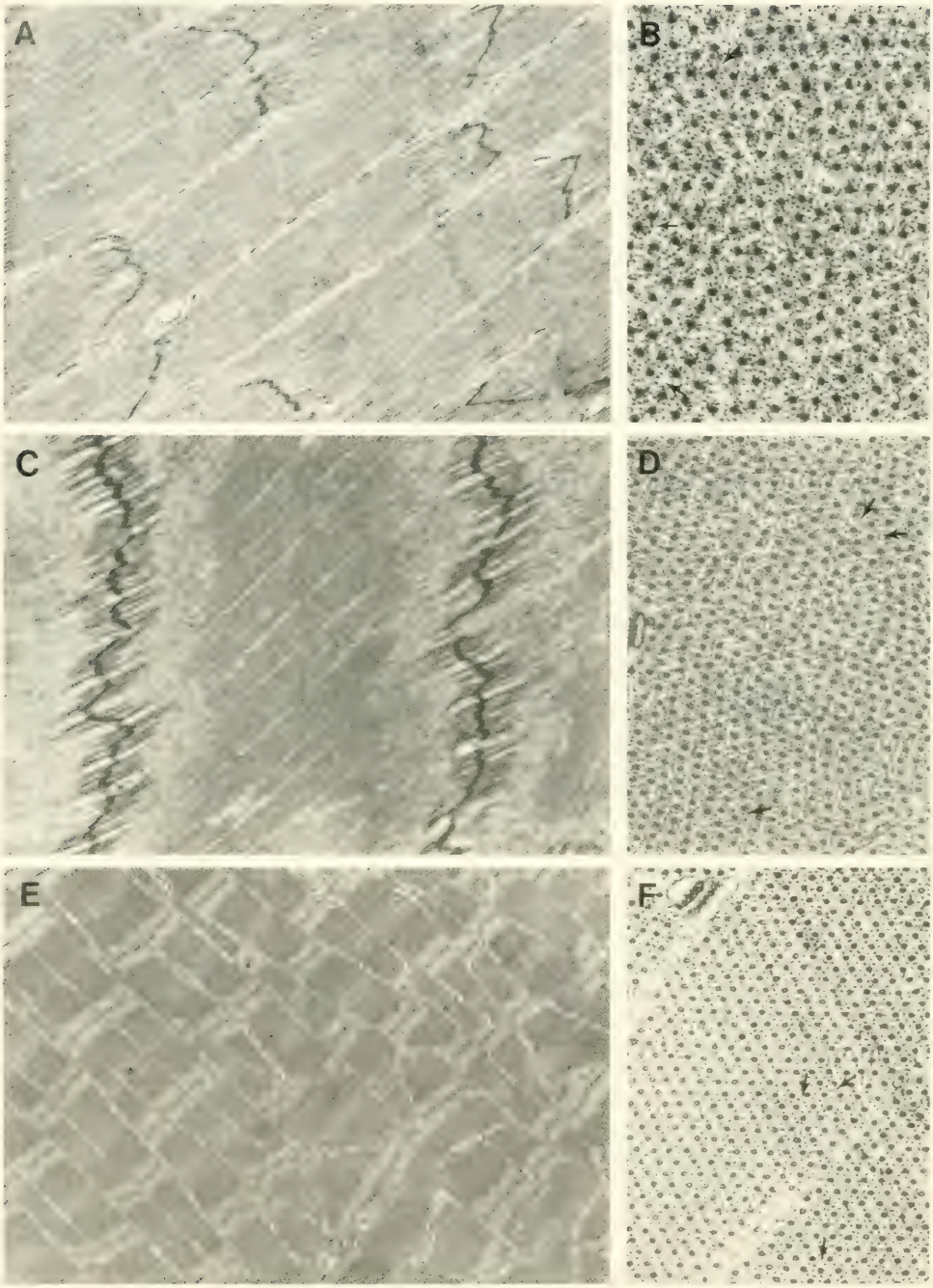

FIGURE 2. Electron micrographs of longitudinal (A, C, E) and cross sections (B, D, F) of claw closer muscle fibers. Sections were taken from a crusher closer muscle (A, B), and a long sarcomere (C, D) and a short sarcomere fiber (E, F) taken from a cutter claw. Orbits where the number of thin filaments surrounding a thick filament can be clearly seen are indicated by arrows. Calibration: 3 μm (A, C, E,) and 0.5 μm (B, D, F,).

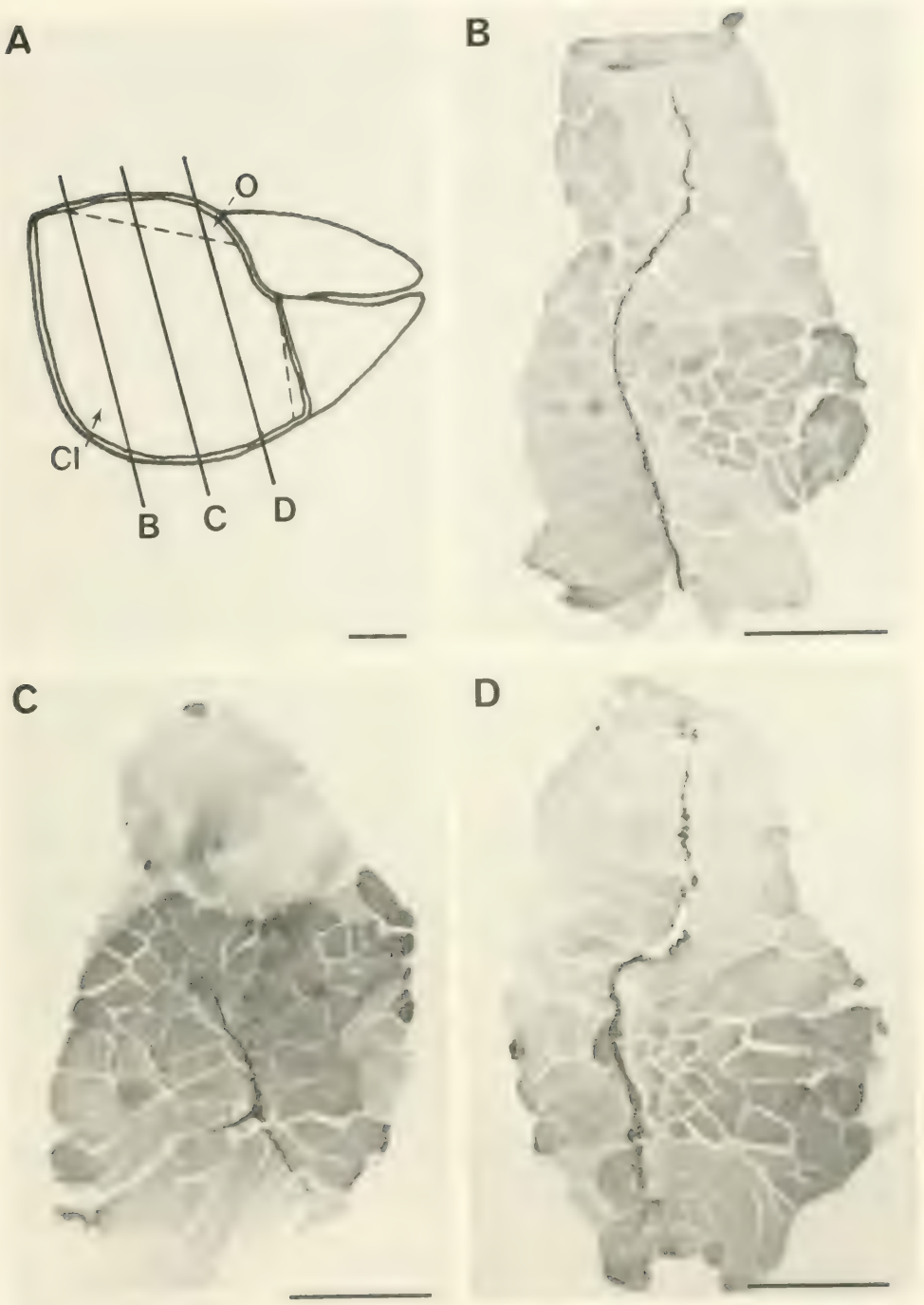

FIGURE 3. Myofibrillar ATPase activity of the cutter closer muscle. The diagram of the cutter claw shows the relative positions of the opener (O) and closer (Cl) muscles in the claw, and the levels at which the sections (B–D) were taken. Note that in all plates the dorsal surface is uppermost and that the shell was removed prior to sectioning of the muscle. Calibration: 100 μm.

et al., 1980). Moreover, sarcomere length measurements made from thoracic limb closer muscles have shown a bimodal distribution of fiber types in lobster (Govind *et al.*, 1981) and in hermit crabs (Table I). These observations suggest that the bimodal distribution of fast and slow fibers may be a common (or even primitive) feature of the crustacean closer muscle. According to this hypothesis, therefore, the enlargement of the right claw of the hermit crab to become the crusher not only involves an increase in the mass of the closer muscle, but also results in the loss of fast fibers. Work on the development of the asymmetric claws of lobsters supports this hypothesis. In larval and early juvenile lobsters, the claws are symmetric and the closer muscle in each claw has fast and slow fibers (Lang *et al.*, 1977b). According to the above hypothesis, therefore, the bimodal distribution of fiber types suggests that during early stages of lobster development the closer muscle fiber types are primitive in both claws. During normal growth the claws become dimorphic and the closer muscles become asymmetric. In the cutter claw closer muscle there is an increase in the number of fast fibers. In the crusher claw closer muscle, by contrast, there is an increase in the number of slow fibers and a decrease (and ultimately a disappearance) of the fast fibers (Ogonowski *et al.*, 1980). Therefore, during normal lobster claw development the specialization of the crusher claw involves skeletal modifications and the loss of fast closer muscle fibers.

Asymmetry has been reported in other regions of the hermit crab besides the claws. There are no abdominal pleopods on the right side, and those that remain on the left have become simplified (Bent and Chapple, 1977a); there has been a concomitant reduction in the number of motor neurons on the right side (Bent and Chapple, 1977b). Similarly, the mass of the deep flexor muscles on the right side is greater than on the left (Marrelli, 1975). It may be argued that reduction of the left side is due to spatial constraints exerted by the right-handed whorls of the gastropod shells in which the crabs live. Similarly, in the case of the dimorphic claws, it seems reasonable that the greater space on the right side favors the location of the larger crusher on this side. However, Chapple (1979) has described dimorphism in the claws as early as the megalops stage, prior to the hermit crab's entry into gastropod shells. In lobster and snapping shrimp claw dimorphism is associated with asymmetries in claw function (Herrick, 1896; Przibram, 1931) and neuromuscular properties (Govind and Lang, 1974; Lang *et al.*, 1977a; Stephens and Mellon, 1979; O'Connor *et al.*, 1982). The present paper reports similar asymmetries in properties of the closer muscles in the dimorphic claws of hermit crabs. Moreover, cursory observations have revealed that there is an asymmetry in the roles of the dimorphic claws during feeding on Slipper shells (*Crepidula*). The major claw is used for holding the shell while the minor claw picks at and transfers the flesh to the mouth.

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LITERATURE CITED

ATWOOD, H. L. 1973. An attempt to account for the diversity of crustacean muscle. *Am. Zool.* **13:** 357–378.

ATWOOD, H. L. 1976. Organization and synaptic physiology of crustacean neuromuscular systems. *Prog. Neurobiol.* **7:** 291–391.

BENT, S. A., AND W. D. CHAPPLE. 1977a. Simplification of swimmeret musculature and innervation in the hermit crab, *Pagurus pollicaris*, in comparison to Macrurans. *J. Comp. Physiol.* **118:** 61–73.

BENT, S. A., AND W. D. CHAPPLE. 1977b. Peripheral and central asymmetry in the swimmeret system of the hermit crab, *Pagurus pollicaris*. *J. Comp. Physiol.* **118:** 75–92.

CHAPPLE, W. D. 1977. Diversity of muscle fibers on the abdominal dorsal superficial muscles of the hermit crab, *Pagurus pollicaris*. *J. Comp. Physiol.* **121:** 395–412.

CHAPPLE, W. D. 1979. Central and peripheral origins of asymmetry in the abdominal motor system of the hermit crab. *Ann. N. Y. Acad. Sci.* **299:** 43–58.

GOVIND, C. K., AND F. LANG. 1974. Neuromuscular analysis of closing in the dimorphic claws of the lobster, *Homarus americanus*. *J. Exp. Zool.* **190:** 281–288.

GOVIND, C. K., T. W. BUDD, AND H. L. ATWOOD. 1981. Fiber composition and innervation patterns of the limb closer muscle in the lobster *Homarus americanus*. *Biol. Bull.* **160:** 69–79.

HERRICK, F. H. 1895. The American lobster; a study of its habits and development. *Bull. U. S. Fish. Comm.* **29:** 149–408.

JOSEPHSON, R. K. 1975. Extensive and intensive factors determining the performance of striated muscle. *J. Exp. Zool.* **194:** 135–154.

LANG, F., W. J. COSTELLO, AND C. K. GOVIND. 1977a. Development of the dimorphic claw closer muscles of the lobster, *Homarus americanus* I. Regional distribution of the muscle fiber types in adults. *Biol. Bull.* **152:** 75–83.

LANG, F., C. K. GOVIND, AND J. SHE. 1977b. Development of the dimorphic claw closer muscles of the lobster, *Homarus americanus* II. Distribution of muscle fiber types in larval forms. *Biol. Bull.* **152:** 382–391.

MARRELLI, J. D. 1975. The morphology and activation of the deep abdominal motor system of the hermit crab *Pagurus pollicaris* (Say), and the homologous relationship to the crayfish deep abdominal motor system. PhD dissertation, University of Connecticut, Storrs.

MELLON, DEF., AND P. J. STEPHENS. 1978. Limb morphology and function are transformed by contralateral nerve section in snapping shrimps. *Nature* **272:** 246–248.

MELLON, DEF., AND P. J. STEPHENS. 1980. Modifications in arrangement of thick and thin filaments in transforming shrimp muscle. *J. Exp. Zool.* **213:** 173–179.

O'CONNOR, K., P. J. STEPHENS, AND J. M. LEFEROVICH. 1982. Regional distribution of muscle fiber types in the asymmetric claws of Californian snapping shrimp. *Biol. Bull.* **163:** 329–336.

O'GONOWSKI, M. M., AND F. LANG. 1979. Histochemical evidence for enzyme differences in crustacean fast and slow muscle. *J. Exp. Zool.* **207:** 143–151.

OGONOWSKI, M. M., F. LANG, AND C. K. GOVIND. 1980. Histochemistry of lobster claw-closer muscles during development. *J. Exp. Zool.* **213:** 359–367.

PRZIBRAM, H. 1931. *Connecting Laws in Animal Morphology*. Univ. of London Press, London. 62 pp.

SCHEIN, H. 1975. Aspects of the aggressive and sexual behavior of *Alpheus heterochelis* Say. *Mar. Behav. Physiol.* **3:** 83–96.

SILVERMAN, H., AND M. P. CHARLTON. 1980. A fast oxidative crustacean muscle: histochemical comparison with other crustacean muscle. *J. Exp. Zool.* **211:** 83–96.

STEPHENS, P. J., AND DEF. MELLON. 1979. Modification of structure and synaptic physiology in transformed shrimp muscle. *J. Comp. Physiol.* **132:** 97–108.

WILSON, E. B. 1903. Notes on the reversal of asymmetry in the regeneration of chelae in *Alpheus heterochelis*. *Biol. Bull.* **4:** 197–210.

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